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Transtympanic injection of a liposomal gel loaded with N-acetyl-L-cysteine: A relevant strategy to prevent damage induced by cochlear implantation in guinea pigs?

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30 **Abstract**

Patients with residual hearing can benefit from cochlear implantation. However, insertion can damage cochlear structures and generate oxidative stress harmful to auditory cells. The antioxidant N-acetyl-L-cysteine (NAC) is a precursor of glutathione (GSH), a powerful endogenous antioxidant. NAC local delivery to the inner ear appeared promising to prevent damage after cochlear implantation in animals. NAC-loaded liposomal gel was specifically designed for transtympanic injection, performed both 3 days before and on the day of surgery. Hearing thresholds were recorded over 30 days in implanted guinea pigs with and without NAC. NAC, GSH, and their degradation products, N,N'-diacetyl-L-cystine (DiNAC) and oxidized glutathione (GSSG) were simultaneously quantified in the perilymph over 15 days in non-implanted guinea pigs. For the first time, endogenous concentrations of GSH and GSSG were determined in the perilymph. Although NAC-loaded liposomal gel sustained NAC release in the perilymph over 15 days, it induced hearing loss in both implanted and non-implanted groups with no perilymphatic GSH increase. Under physiological conditions, NAC appeared poorly stable within liposomes. As DiNAC was quantified at concentrations which were twice as high as NAC in the perilymph, it was hypothesized that DiNAC could be responsible for the adverse effects on hearing.

Keywords

Glutathione, hyaluronic acid, inner ear, local drug delivery, N,N'-diacetyl-L-cystine, oxidative stress

50 **Abbreviations**

ABR, auditory brainstem response; CI, cochlear implanted; Chol, cholesterol; DiNAC, N,N'-diacetyl-L-cystine; D_h , hydrodynamic diameter; DL, drug loading; DLS, dynamic light scattering; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPE-PEG₂₀₀₀, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-poly(ethyleneglycol)-2000]; EE, encapsulation efficiency; EPC, egg phosphatidylcholine; GSH, L-glutathione; GSSG, oxidized L-glutathione; HA, hyaluronic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LC/MS: liquid chromatography/mass spectroscopy; Lip, unloaded DPPC-based PEGylated liposomes; NAC, N-acetyl-L-cysteine; NAC-Lip, NAC-loaded DPPC-based PEGylated liposomes; MRM, Multiple Reaction Monitoring; PEG, polyethylene glycol

1. Introduction

The cochlear implant is currently the most successful neural prosthesis with more than 300 000 implantations per year worldwide (NIDCD, 2016). The device allows patients with severe to profound sensorineural hearing loss to perceive sounds (Lenarz, 2017). The auditory neurons are directly stimulated through electrodes placed in the cochlea. Nowadays, patients with residual hearing can benefit from the so-called electroacoustic cochlear implant, with both electrical and acoustical stimulation of the inner ear (Lenarz, 2017). Because the loss of acoustic stimulation reduces quality-of-life for these patients (Bas et al., 2012), preserving residual hearing during insertion of the electrode array in the cochlea is an important issue.

The soft surgery technique contributed to limiting electrode array-induced trauma on the surviving auditory cells (Lenarz, 2017). Robot-assisted insertion (Vittoria et al., 2021) or motorized insertion (Mamelle et al., 2017) may provide additional protection. Moreover, therapeutic supplementation could also be useful to stop the deleterious mechanisms induced by electrode array insertion. Indeed, the trauma initiates an inflammatory response and an oxidative stress that are harmful to auditory cells (Bas et al., 2012). High concentrations of reactive oxygen species (*i.e.* hydrogen peroxide, superoxide) were measured in the inner ear after cochlear implantation in humans (Ciorba et al., 2010). Hydrogen peroxide may represent an intermediate molecule between hydroxyl radical and superoxide, the latter being less reactive. They played a key role in cochlear implant trauma (Bas et al., 2012). However, the duration of this mechanism is unknown. Unlike corticoids acting on the inflammation (Borden et al., 2010; Eastwood et al., 2010a; Wang et al., 2011; Honeder et al., 2016; Mamelle et al., 2017), antioxidants have been poorly evaluated in the inner ear applications (Eastwood et al., 2010b; Chang et al., 2017) although they appear particularly relevant for this therapeutic indication.

N-Acetyl-L-cysteine (NAC) has a direct antioxidant effect on reactive oxygen species (hydroxyl radical, hydrogen peroxide) and is also a precursor for glutathione (GSH) synthesis, a powerful endogenous antioxidant (Atkuri et al., 2007). GSH can convert hydroxyl radical and hydrogen peroxide into molecules of water. For several decades, NAC has commonly been used against acetaminophen overdose or as a mucolytic agent and is described as safe in humans (Atkuri et al., 2007).

In the literature, the results for administration of NAC were variable depending on the model used. In humans, its administration by transtympanic injection (through the tympanic

membrane to fill the middle ear cavity) was evaluated to limit cisplatin ototoxicity. NAC
95 (100 mg/mL) protected hearing thresholds by 10 dB at 8 kHz (Riga et al., 2013). In patients
with important hearing loss (2 out of 11 patients), NAC protected auditory function from
cisplatin ototoxicity (Yoo et al., 2014). However, the time of contact of the solution with the
round window membrane that separates the middle from the inner ear was less than
100 30 minutes. In animals, results on audition were mitigated by using NAC to prevent cisplatin
ototoxicity (Choe et al., 2004; Nader et al., 2010; Saliba et al., 2010; Mohan et al., 2014), and
damage from cochlear implantation or noise trauma (Eastwood et al., 2010b). Choe et al.
(2004) demonstrated protection of the auditory function by NAC while others showed mixed
outcomes (Eastwood et al., 2010b; Saliba et al., 2010), no effect (Mohan et al., 2014) or
pronounced hearing loss in the treated group (Eastwood et al., 2010b; Nader et al., 2010).
105 Possible reasons for these failures were because NAC solutions did not respect the standard
specifications for intratympanic administration: NAC solutions were either hyperosmolar
(Nader et al., 2010; Saliba et al., 2010) or had an acidic pH (2.5) (Eastwood et al., 2010b).
Moreover, as NAC is sensitive to light, oxygen, temperature, pH, and heavy metals (Caritá et
al., 2020), its degradation might also have affected its biological activity.

110 Transtympanic injections are widely used in the clinic to maximize drug concentration in
the inner ear and are described as the safest and most reliable local route of administration
(for reviews, see El Kechai et al., 2015a and Jaudoin et al., 2021). As appropriate
formulations are not available on the market (Le Prell, 2019), liquid dosage forms are used
off-label in clinical practice. However, they are rapidly eliminated by the Eustachian tube
115 after transtympanic injection (Plontke et al., 2008). Once injected into the middle ear, drugs
must diffuse through the round window membrane to reach the perilymph (inner ear fluid).
This diffusion is highly dependent on the drug physicochemical characteristics (El Kechai et
al., 2015a; Salt et al., 2016; Jaudoin et al., 2021). Thus, repeated injections are needed
reducing patient compliance and comfort. Recently, hybrid formulations of nanocarriers
120 incorporated in gels have been developed (Jaudoin et al., 2021). They offer many advantages
over classic formulations: increased residence time in the middle ear, sustained drug delivery
and passage and/or accumulation of nanocarriers through the round window membrane.

In our group, we developed an innovative drug delivery system that met the requirements
for prolonged inner ear delivery: a hyaluronic acid (HA) gel containing polyethylene glycol
125 (PEG)-coated liposomes (El Kechai et al., 2016). This HA liposomal gel was easily injected
into the middle ear (El Kechai et al., 2015b). Due to non-thixotropic and viscoelastic

properties, its elimination by the Eustachian tube was reduced. Liposomes migrated from the gel to accumulate in the round window membrane, acting as a drug reservoir (El Kechai et al., 2016). Dexamethasone phosphate was released into the perilymph from this formulation for up to 1 month at therapeutic concentrations, which was longer than previously reported with other gels (5–10 days) (Borden et al., 2010; Wang et al., 2011). This drug delivery system was well-tolerated in a guinea pig model as attested by the absence of any deleterious effect on both hearing function and the middle ear mucosa (El Kechai et al., 2016).

The aim of the present study was to deliver an antioxidant, NAC, to the inner ear to prevent damage induced by cochlear implantation. Firstly, we designed a HA liposomal gel loaded with NAC (HA-NAC-Lip gel) that complied with the specifications for transtympanic injection (pH, osmolality, no preservatives). Secondly, the effect of HA-NAC-Lip gel was evaluated in an implanted guinea pig model receiving two injections (3 days before and on the day of surgery). The effect of this formulation on hearing was also checked in non-implanted animals. Finally, the ability of this system to release NAC in the perilymph and stimulate GSH synthesis was assessed in normal-hearing guinea pigs.

2. Materials and methods

2.1. Materials

N-Acetyl-L-cysteine (NAC) (Ph. Eur. grade) was provided by Inresa (Bartenheim, France), N,N'-diacetyl-L-cystine (DiNAC) by Bertin Pharma (Montigny-le-Bretonneux, France), N-acetyl-L-(cysteine-¹³C₃,¹⁵N) by Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, purity 99.9%) and egg phosphatidylcholine (EPC, purity 96%) were kindly given by Lipoid GmbH (Ludwigshafen, Germany). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-poly(ethyleneglycol)-2000] (DSPE-PEG₂₀₀₀) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Sodium hyaluronate (HA, $M_w = 1.14 \pm 0.05$ MDa, $\bar{D} = 1.13$, determined by size-exclusion chromatography, batch A0375841, purity 95%) was provided by Acros Organics (Geel, Belgium). Perchloric acid (70%) from Merck (Darmstadt, Germany) was used for the Bartlett test. L-Glutathione (GSH), oxidized L-glutathione (GSSG), glutathione-(glycine-¹³C₂,¹⁵N) trifluoroacetate, Cholesterol (Chol), Tween[®] 80, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphoric acid, trichloroacetic acid, L-ascorbic acid, 0.65 mM phosphorus standard solution and ammonium molybdate were

purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Milli-Q water was used (Millipore, Molsheim, France). All other chemicals were of analytical grade.

160 **2.2. Stability of NAC solutions**

To prevent NAC degradation from light and oxygen, 30 g/L and 5 g/L NAC solutions (HEPES buffer, 10 mM, pH 7.4) were prepared in amber glassware and under argon. Previously, oxygen was removed from all buffers and NAC solutions using a modified argon purging method (Butler et al., 1994). NAC solutions and buffers were bubbled with argon
165 under stirring until the dissolved oxygen measured by an oximeter (Inlab OptiOx, Mettler Toledo, Schwerzenbach, Switzerland) was < 1 mg/L. The stability of NAC solutions (n = 3) was studied over a 1-month period under storage conditions (4 °C, argon) and conditions similar to those of the middle ear cavity (37 °C, normal atmosphere).

2.3. Preparation of liposomes

170 Unloaded DPPC-based PEGylated liposomes (Lip) were prepared by the thin film hydration method (Bangham et al., 1965) for Transwell® liposome release and *in vivo* studies. Lipids dissolved in chloroform solutions were sampled with a Hamilton syringe (Bonaduz, Switzerland) in appropriate amounts to reach the theoretical final lipid concentration of 90 mM and mixed in an amber glass balloon. The lipid film composed of DPPC:Chol:DSPE-
175 PEG₂₀₀₀ (60:35:5 mol%) was obtained using a rotary evaporator (BÜCHI R-124, Villebon-sur-Yvette, France) under vacuum and hydrated with HEPES/NaCl buffer (10/115 mM, pH 7.4) by heating at 50 °C for 3 min using a waterbath, followed by three cycles of vortex (1 min) and heating (1 min). The suspension was extruded on 0.2 µm polycarbonate membranes (Millipore, Molsheim, France) with a Lipex extruder (Whitley, Vancouver,
180 Canada) at 50 °C, until the mean hydrodynamic diameter (D_h) was between 145 and 155 nm (*see section 2.4.1*).

EPC-based PEGylated liposomes (EPC:Chol:DSPE-PEG₂₀₀₀, 60:35:5 mol%) were also prepared for Transwell® release studies (*see section 2.7.3*) following the protocol of an earlier study (El Kechai et al., 2016).

185 NAC-loaded PEGylated liposomes (NAC-Lip) were prepared using only amber glassware and the same protocol as for Lip, except that the lipid film (DPPC:Chol:DSPE-PEG₂₀₀₀) was hydrated with 3 mL of 30 mg/mL NAC solution in HEPES buffer (10 mM, pH 7.4, dissolved oxygen < 1 mg/L). To prevent NAC oxidation, NAC-Lip were manufactured under an inert

atmosphere (nitrogen or argon). After extrusion, non-encapsulated NAC was eliminated by
190 dialysis with repeated changes of medium for 8 h at 4 °C. A Gebaflex® dialysis bag with a
molecular weight cut-off between 12 and 14 kDa (Interchim, Montluçon, France) was used
against HEPES/NaCl buffer (10/115 mM, pH 7.4, dissolved oxygen < 1 mg/L). All of the
samples were protected from light and oxygen.

2.4. Liposome characterization

195 2.4.1. Size and zeta (ζ) potential determination

Hydrodynamic diameter (D_h) and ζ potential of the liposomes (diluted to 2 mM [lipids] in
Milli-Q water) were determined in triplicate at 25 °C using a Zetasizer Nano ZS (Malvern
Panalytical Ltd, Malvern, Worcestershire, UK). D_h was measured by dynamic light scattering
(DLS) at a backscattering angle of 173°, and a refractive index of 1.33 and 1.45, respectively,
200 for dispersant and material. The surface potential was assessed by the ζ potential at a count
rate > 100 kcps and conductivity around 0.3 mS.cm⁻¹.

2.4.2. Lipid quantification

Two lipid quantification methods were used: Bartlett and enzymatic assays. The enzymatic
assay was faster but was not accurate for DPPC-based suspensions. Furthermore, it relied on
205 an oxidoreduction reaction, which was not suitable for NAC-loaded liposomes.

– *Bartlett assay*

The concentration of DPPC and DSPE-PEG₂₀₀₀ in liposome suspensions was determined
using a modified Bartlett assay (Barenholz and Amselem, 1993) after extrusion (Lip), dialysis
(NAC-Lip), or in a Transwell® liposome release study. A calibration curve was made with a
210 phosphorus standard solution. The linear working range of the method corresponded to 0.75–
12.5 mM of liposomes. If necessary, liposome suspensions were diluted, and water was
evaporated in separate glass tubes at 100 °C in duplicate on a heating block. Then 300 μ L of
perchloric acid was added and the samples were heated at 190–200 °C for 25 min. After
cooling at ambient temperature, 1 mL of deionized Milli-Q water, 400 μ L of 1.25% (w/w)
215 ammonium molybdate and 400 μ L of fresh 5% (w/v) ascorbic acid solutions were mixed with
the samples. After heating the tubes for 5 min at 100 °C, absorbance was measured at 820 nm
(Lambda 25, UV–Vis spectrometer, PerkinElmer, Waltham, MA, USA). The phosphate
inorganic content of liposome samples was determined from the calibration curves. The total

amount of lipids in liposome suspensions was then extrapolated assuming that the lipid
220 composition remained unchanged during liposome preparation.

– *Enzymatic assay*

EPC concentration was quantified by an enzymatic phospholipid assay (Biolabo SA,
Maizy, France) after extrusion. Suspensions were diluted to perform measurements in the
225 validity range of the assay (0.22–10.75 mM). A 10 μ L aliquot of sample was incubated with
1 mL of enzyme reagent for 10 min in a waterbath at 37 °C (n = 2). Absorbance was
measured at 500 nm (Lambda 25 UV–Vis spectrometer, PerkinElmer, Waltham, MA, USA).
On the assumption that the lipid ratio did not change during the different steps of liposome
preparation, the total amount of lipids was calculated from the EPC concentration (Eq. 1):

$$[Lipids] = \frac{A_{measured}}{A_0} \times \frac{Y [Lipids]_0}{M_{EPC} f_{EPC}} \times 10^3 \quad \text{Eq. 1}$$

230 with A_0 and $[Lipids]_0$, respectively, the absorbance and lipid concentration (mM) of a standard
solution, M_{EPC} and f_{EPC} , respectively, the molar mass (g/mol) and the molar fraction of EPC
(mol/mol) in the EPC formulation, and Y the dilution factor of the liposomes.

2.4.3. Drug loading and efficiency

After dialysis, the amount of NAC encapsulated in NAC-Lip (n = 3) was determined by
235 high-performance liquid chromatography (HPLC) (*see section 2.5*) after solubilization of
50 μ L of NAC-Lip in 500 μ L of 5% Tween[®] 80. Effective destruction of the vesicular shape
of NAC-Lip was assessed by DLS (*data not shown*). The encapsulation efficiency (EE%) (Eq.
2), and the drug loading (DL%) (Eq. 3), were calculated:

$$EE\% = \frac{\text{amount of NAC entrapped in NAC-Lip (g)}}{\text{initial amount of NAC (g)}} \times 100 \quad \text{Eq. 2}$$

$$DL\% = \frac{\text{amount of NAC entrapped in NAC-Lip (g)}}{\text{amount of lipids (g)}} \times 100 \quad \text{Eq. 3}$$

2.5. Quantification of NAC and degradation product DiNAC by HPLC–UV

240 For determination of drug loading and encapsulation efficiency, stability and release
studies, NAC and its degradation product, DiNAC, were quantified by HPLC-UV (Ph. Eur.
10.2, 2020). NAC and DiNAC were separated using a Gemini C18 column (3 μ m, 110 \times
4.6 mm) equipped with a C18 (4 \times 2 mm) guard column maintained at 30 °C and detected on

a Jasco UV–1570 detector (Jasco Corp., Tokyo, Japan) at 220 nm. The eluent was composed
245 of acetonitrile/water (3/97 v/v) acidified at pH 2.4 with phosphoric acid, degassed in-line. A
flow rate of 1.2 mL/min was used (515 HPLC pump, Waters, Milford, MA, USA).
Calibration curves (14 points) were obtained in triplicate with freshly prepared NAC and
DiNAC solutions (HEPES/NaCl 10/115 mM buffer, pH 7.4, dissolved oxygen < 1 mg/mL).
250 Samples were protected from light and kept at 10 °C under argon (717plus Autosampler,
Waters, Milford, MA, USA). NAC and DiNAC were eluted at 2.4 and 8 min, respectively, for
an injection volume of 20 µL. Area under the curve (AUC) was measured using Azur v4.6.0.0
software. The method showed satisfactory linearity for NAC between 9.9 and 1500 mg/L
($R^2 = 0.9999$) and DiNAC between 18.2 and 2500 mg/L ($R^2 = 0.9999$). The detection limits of
NAC and DiNAC were, respectively, 9.9 and 18.2 mg/L based on the standard deviation of
255 the response and the slope (ICH Q2 R1). The quantification limits of NAC and DiNAC were,
respectively, 30.1 and 55.2 mg/L. The use of Tween[®] 80 did not impact the quantification of
NAC and DiNAC (*data not shown*).

2.6. Stability of NAC-Lip and release

The stability of NAC-Lip (n = 3) was assessed over 7 days by measuring NAC and DiNAC
260 in the suspension under storage conditions (4 °C, argon) or *in vivo*-mimicking conditions
(37 °C, normal atmosphere). NAC release from NAC-Lip was monitored over time using
ultrafiltration. For each batch, a sample of 1 mL was kept either under storage or *in vivo*-
mimicking conditions. At each time point (days 1, 3 and 7), 100 µL of the suspension was
sampled. A 50 µL aliquot was used for NAC and DiNAC quantification. The NAC released
265 from NAC-Lip was then separated from the encapsulated NAC using 0.5 mL Amicon
Ultracentrifugal filters (M_w cut-off 100 kDa, Millipore, Molsheim, France). After a 1:10
dilution in HEPES/NaCl buffer, samples were centrifuged for 20 min at 12,000 g (Eppendorf
minispin[®], Hamburg, Germany). A second centrifugation was performed for 10 min at
12,000 g after dilution with 100 µL of buffer. NAC and DiNAC were quantified in the
270 retentate by HPLC (*see section 2.5*), after solubilization in 5% Tween[®] 80, and in the filtrate.
The procedure was performed on ice, under argon and with amber glassware.

2.7. Preparation and characterization of HA liposomal gels

HA liposomal gels were prepared by dissolving HA (1.5% w/v) in the liposome
suspensions. Lip and NAC-Lip were adjusted, respectively, at 80 and 74 mM of final lipid
275 concentration, corresponding to 4.5 g/L of NAC for NAC-Lip. HA liposomal gels (HA-Lip

and HA-NAC-Lip) were immediately vortexed for 10 min, then maintained at room temperature for 1 h and manually stirred for 1 min. Bubbles were removed using a vacuum pump and normal atmosphere was replaced by argon. Gels were stored at 4 °C for at least 12 h before analysis or *in vivo* administration. Formulations were protected from light throughout the process. Aliquots of 1.5 mL, 2 mL and 3 mL of HA liposomal gels were prepared, respectively, for *in vivo*, Transwell® liposome release and rheological studies.

2.7.1. Osmolality measurements

HA liposomal gel (HA-Lip and HA-NAC-Lip) osmolality was measured with an osmometer (Roebbling, Berlin, Germany), by the freezing point depression determination.

2.7.2. Rheological behavior of HA liposomal gels

Flow measurements were performed in triplicate on three batches of HA-EPC-based PEGylated liposomes, HA-Lip and HA-NAC-Lip using an ARG2 rotational rheometer (TA Instruments, New Castle, DE, USA) equipped with an aluminum cone/plate geometry (diameter 4 cm, angle 1° and cone truncation 28 µm). Measurements were made under steady-state conditions. After 2 min of equilibration at 37 °C, the shear rate was increased from 0.01 to 1000 s⁻¹, maintained at 1000 s⁻¹ for 2 min, and then decreased to 0.01 s⁻¹. The viscosity curves were fitted according to the Williamson equation (Eq. 4) (Milas et al., 2002) using TRIOS software (TA Instruments, Waters LCC, New Castle, DE, USA) to determine the zero-shear rate viscosity η_0 (Pa.s).

$$\eta = \frac{\eta_0}{1 + (k\dot{\gamma})^n} \quad \text{Eq. 4}$$

with η the viscosity (Pa.s), k the consistency (s), $\dot{\gamma}$ the shear rate (s⁻¹) and n the power law index. Such a model is suited to describe a shear-thinning behavior.

2.7.3. Liposome release from HA liposomal gels

Lip release from HA-Lip gels was compared to the release of EPC-based PEGylated liposomes from gels. This latter system, previously studied *in vivo* by El Kechai et al. (2016) to administer dexamethasone phosphate, was used as a reference. The migration of liposomes within the gel was evaluated on Transwell® Costar® multiple well plates with polycarbonate membrane inserts (Corning, Kennebunk, ME, USA) at 37 °C. The pore size of the polycarbonate membrane (thickness 10 µm, surface area 0.33 cm²) of the Transwell® insert

was 3 μm (Fig. 1). The membrane allowed the rapid diffusion of the liposomes when in suspension, without changing their size distribution, meaning that the membrane was non-limiting (*data not shown*).

Aliquots of 150 μL of HA-Lip and 500 μL of HEPES/NaCl buffer (10/145 mM, 7.4) with equivalent osmolalities were placed, respectively, in the upper and lower compartments of the well to limit water fluxes from one compartment to the other due to osmotic pressure. The plate of 12 wells (diameter 6.5 mm) was closed with adhesive tape to avoid evaporation and stored in an oven at 37 $^{\circ}\text{C}$. At different time points (0.25, 1, 1.25, 2, 2.25, 3, 3.25, 7, 8, 10, 14 and 21 days), the entire content of the lower compartment was removed and weighed (*detailed protocol in Fig. 1*).

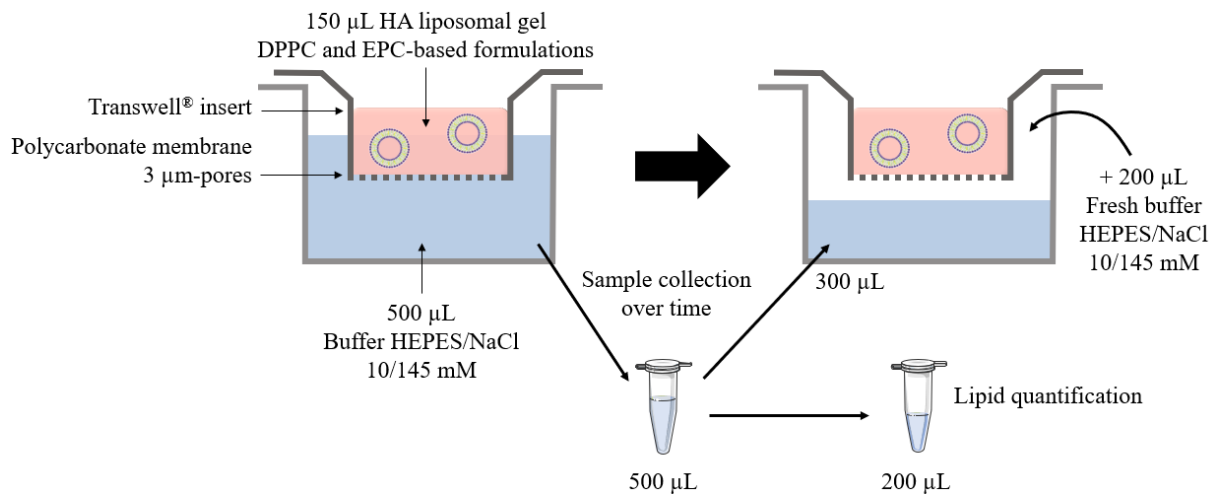


Fig. 1. Sampling protocol on Transwell[®] model.

A 300 μL aliquot of the original content was replaced in the lower compartment and 200 μL was kept at 4 $^{\circ}\text{C}$ for lipid quantification (*see section 2.4.2*). Then, the content of the lower compartment was adjusted to 500 μL with fresh buffer. The cumulated amount of lipids released over time was expressed as a percentage of the total amount of lipids (in moles) added to the upper compartment at the initial time. Results were expressed as mean \pm standard deviation of three wells.

2.8. *In vivo* study

2.8.1. *Animals*

Thirty-two male Hartley albino guinea pigs (300–500 g, Charles River, L'Arbresle, France), with normal hearing thresholds were used. Animal care and experimental procedures were conducted in accordance with European legislation and approved by the local ethics committee (CEEA no. 26) of Université Paris-Saclay (permission Apafis # 11900-

2017102312027309 v1). Experiments were conducted after 2 weeks of acclimatization for the guinea pigs.

330 All experiments were performed under general anesthesia (xylazine: 4 mg/kg, Rompun™, Bayer, Leverkusen, Germany; ketamine: 60 mg/kg, Virbac, France; one intramuscular injection). To ease the recovery of the animal from anesthesia, the antidote of xylazine (atipamezole, 0.15 mg/kg, Antisedan®, Orion, Espoo, Finland) was administered intramuscularly at the end of each surgical procedure, except after perilymph sampling for
335 which animals were euthanized with pentobarbital (110 mg, Dolethal®, Vetoquinol, Lure, France).

2.8.2. Study design

After initial determination of the auditory thresholds by measurement of the auditory brainstem response (ABR) (*see section 2.8.5*), animals were assigned to different groups:

- 340 – **CI HA-Lip group:** Effect of cochlear implantation on hearing function.
- **CI HA-NAC-Lip group:** Effect of HA-NAC-Lip on hearing in a cochlear implantation model.
- **HA-NAC-Lip group:** Hearing effect of HA-NAC-Lip and quantification of NAC, GSH and their degradation products, DiNAC and GSSG, in the perilymph in the absence of
345 cochlear implantation.
- **Endogenous group:** Determination of GSH and GSSG endogenous concentrations in the perilymph.

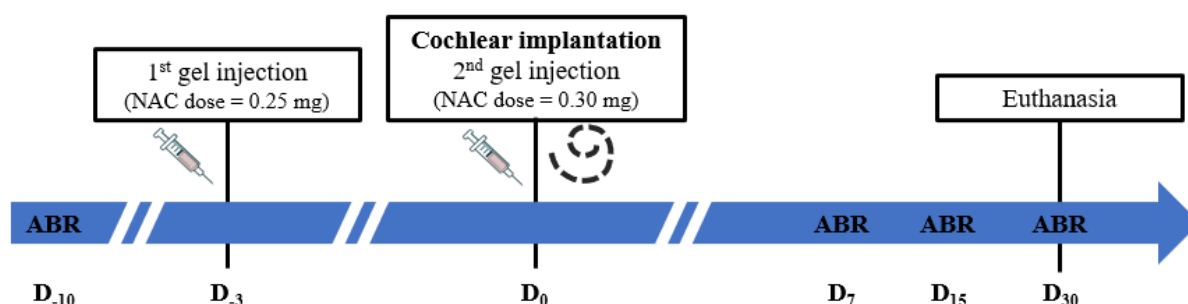
To ensure that NAC was already available in the scala tympani when the trauma was induced by electrode array insertion, one gel injection was performed 3 days before (D₋₃) the
350 day of implantation (D₀). A second injection was administered at D₀ after removal of the remaining gel and before cochlear implantation. The study design is presented in Fig. 2.

Cochlear implantation

CI HA-Lip group (8 animals)

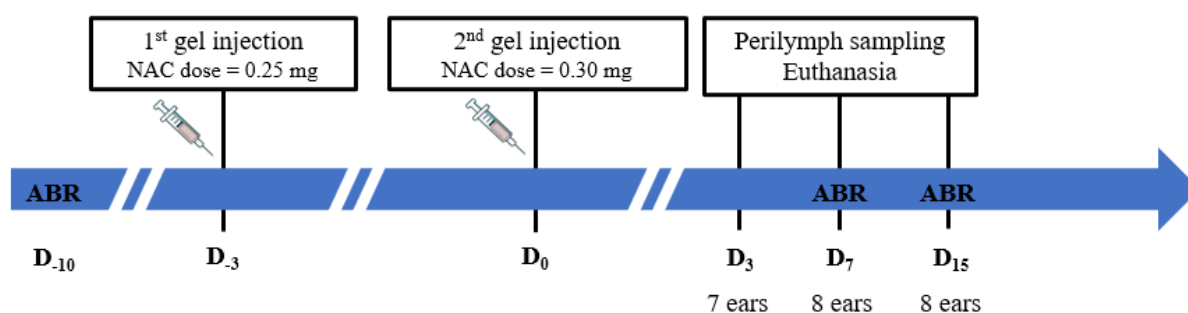
CI HA-NAC-Lip group (6 animals)

} *Contralateral ear cophosed*



Tolerance and quantification of NAC, DiNAC, GSH and GSSG in the perilymph

HA-NAC-Lip group (12 animals), *both ears used*



Endogenous levels of GSH and GSSG in the perilymph

Endogenous group (6 animals), *both ears used*

Perilymph sampling
Euthanasia

ABR = Auditory Brainstem Response measurement

Fig. 2. Experimental protocols for cochlear implantation, tolerance and quantification of NAC, DiNAC, GSH and GSSG in the perilymph without cochlear implantation, and endogenous concentrations of GSH and GSSG in the perilymph.

2.8.3. Injection procedure for HA liposomal gels

HA liposomal gel (HA-NAC-Lip or HA-Lip) injections in the middle ear were performed under a binocular loupe at D₋₃ and D₀, using a Luer-lock[®] Fisherbrand 3P 1 mL syringe (Fisher Scientific, Illkirch, France) equipped with a 29 G Spinocan needle (diameter = 0.35 mm, length = 88 mm, B. Braun Medical S.A., Diegem, Belgium). The syringe filled with HA liposomal gel was weighed before and after injection, to estimate the amount of gel administered.

- At D₋₃, HA-NAC-Lip or HA-Lip (113 ± 31 μL, n = 38 ears, corresponding to 0.25 mg of NAC) was injected through the tympanic membrane into the middle ear cavity.
- 365 • At D₀, a retro-auricular approach to the bulla was performed after shaving and cleaning the back of the head with povidone-iodine (Betadine[®], Meda Pharma, Solna, Switzerland). Lidocaine hydrochloride diluted to 0.5% (0.8 mg/kg, AstraZeneca, London, UK) was injected into retro-auricular subcutaneous tissue. After a retro-auricular incision, muscles were gently pushed aside from the bone and the bulla was carefully opened using
370 a 2 mm diamond bur to drill the bone (NSK, Nakashima, Japan). After visual evaluation of the amount of HA liposomal gel remaining in the middle ear, the bulla was washed with saline solution to allow cochlea and round window visualization. HA liposomal gel (138 ± 28 μL, n = 38 ears, corresponding to 0.30 mg of NAC) was then injected to completely fill the middle ear. Muscles and skin were sutured with 3.0 Vicryl (Ethicon, Cincinnati, OH, USA).
375

2.8.4. Cochlear implantation procedure

Sterile electrode arrays specifically designed for guinea pigs were manufactured by Oticon Medical (Vallauris, France). They were composed of four couples of platinum-iridium electrodes and wires included in a medical grade silicon array. The outer diameter was
380 0.3 mm. The total length of the array measured 7 mm, but the insertion was stopped at 4 mm-depth in all procedures, corresponding to the fourth electrode located next to the cochleostomy.

An experienced otologist surgeon performed the cochlear implantations. Before surgery, anesthetized guinea pigs received a subcutaneous injection of buprenorphine at 0.04 mg/kg
385 (Buprecare, Eucuphar, Oostkamp, Belgium). A retro-auricular approach to the bulla was performed (*see section 2.8.3*), the basal turn of the cochlea was exposed, and a cochleostomy was performed 1.5 mm under the round window ridge with a 0.6 mm micro perforator (Karl Storz, Tuttlingen, Germany). Hyaluronic acid solution without liposomes (1.5% w/v) was then placed over the cochleostomy to prevent any perilymph leakage before cochlear insertion
390 as in clinical routine (Lenarz, 2017). The electrode array was manually inserted using micro-forceps. Before suturing the skin, the bulla was completely filled with HA-NAC-Lip or HA-Lip, as outlined in the injection procedure. To avoid osseous transcranial sound conduction, the contralateral ear was cophosed; a large cochleostomy was performed and small muscle

fragments were introduced into the cochlea to obtain total sensorineural hearing loss. After
395 surgery, ibuprofen (5 mg/kg, Advil[®], Pfizer, Paris, France) was given *per os*.

2.8.5. Auditory Brainstem Response measurements

Animal hearing was recorded 1 week before surgery and at postoperative days depending
on the protocol (Fig. 2). A soundproofed cab designed for small animals and enclosed for
radiofrequency electromagnetic screening purposes was used for hearing recordings
400 (dimensions 48/33/51 cm, RT conception, Clermont-Ferrand, France). Clicks (centered at
2 kHz) and tone bursts (4, 8, 16, and 32 kHz) of 100 ms duration were delivered with a high
frequency transducer (Otophylab, RT-Conception Ltd., Clermont-Ferrand, France) connected
to an external ear canal probe. Needle electrodes (Medtronic Xomed, Jacksonville, FL, USA)
405 were placed subcutaneously in the leg, the vertex, and the retro-auricular side of the analyzed
ear. Hearing thresholds were visually determined with 5 dB decreasing increments using RT-
Lab software v1.1.5.0 (Echodia, Clermont-Ferrand, France). The hearing threshold
corresponded to the minimum intensity for which one or several ABR waves were clearly
recognized and reproducible on two different recordings. The accuracy of ABR threshold
measurement was ± 5 dB.

2.8.6. Quantification of NAC, DiNAC, GSH and GSSG in the perilymph

– Perilymph sampling

Just before euthanasia, the basal turn of the cochlea was exposed by a retro-auricular
procedure (*see section 2.8.3*). The persistence of the HA liposomal gel in the middle ear was
systematically assessed by macroscopic observation of the amount of remaining HA
415 liposomal gel. The bulla was rinsed several times with saline solution to eliminate any gel
traces. The bone was thinned with a 0.4 mm micro-perforator 2 mm under the round window
niche to access the perilymph. Then, a controlled puncture was completed with a homemade
microcapillary pipette (distal diameter $< 10 \mu\text{m}$) fixed to a micromanipulator. Less than $2 \mu\text{L}$
of perilymph – to avoid sample contamination with cerebrospinal fluid – was collected by a
420 slow aspiration. The sample volume was accurately measured after the transfer to a calibrated
 $10 \mu\text{L}$ microcap tube. Perilymph samples contaminated with blood were excluded, as GSH
concentration was expected to be higher in blood than perilymph (Giustarini et al., 2016).
Samples were immediately treated and stored at $-80 \text{ }^\circ\text{C}$ under argon.

425 – *Liquid chromatography/mass spectroscopy (LC/MS)*

NAC, GSH and their respective degradation products, DiNAC and GSSG, were quantified in perilymph samples by an Acquity UPLC (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Each substance and two deuterated internal standards, N-acetyl-L-(cysteine-¹³C₃,¹⁵N) and glutathione-(glycine-¹³C₂,¹⁵N) trifluoroacetate, were eluted on a Gemini C18 column (3 μm, 100 x 2 mm) equipped with a C18 guard column (4 x 2 mm) (Phenomenex, Torrance, CA, USA). The mobile phase was composed of methanol/water (3/97 v/v) with 0.1% formic acid, with a gradient starting at 4 min increasing the level of methanol to reach 20% v/v at 8 min. The flow was set at 0.25 mL/min. Source and gas desolvation temperatures were fixed, respectively, at 120 °C and 350 °C and desolvation and cone gas flow, respectively, at 500 L/h and 50 L/h. Capillary and cone voltage were established at 2.9 kV in negative mode and 3.2 kV in positive mode. Extractor voltage and radiofrequency lens were maintained at 3 V and 0.1 V, respectively. Cone voltage was optimized for each ion to obtain the most intense signal. The collision gas was argon at a pressure maintained near $4.5 \cdot 10^{-3}$ mbar in the collision cell. The acquisition mode was the Multiple Reaction Monitoring (MRM) mode and data were processed using MassLynx v4.1 software (Waters, Milford, MA, USA). Quantification was performed by integration of the area under the peak of interest within the MRM chromatogram. All substances were quantified in negative electrospray mode, apart from GSH for which positive electrospray was also performed. MRM was optimized to follow the transitions as shown in Table 1.

450

Table 1. Parameters of the LC/MS method for NAC, DiNAC, GSH, GSSG and internal standards

Substance	Abbreviation	RT (min)	Adduct	Cone voltage (V)	m/z parent → daughter	Collision energy (eV)	LOD pmol/μL	LOQ pmol/μL
N-acetyl-L-cysteine	NAC	3.2	[M-H] ⁻	21	162.1 → 84.1	12	0.6	1.2
N-acetyl-L-(cysteine- ¹³ C ₃ , ¹⁵ N)		3.2	[M-H] ⁻	21	166.1 → 87.1	12	–	–
N,N'-diacetyl-L-cystine	DiNAC	6.7	[M-H] ⁻	24	323.2 → 128.2	14	0.03	0.06
L-glutathione	GSH	1.9	[M-H] ⁻	25	308.4 → 179.2	15	0.7	1.6
		1.9	[MH] ⁺	25	306.2 → 143.2	15		
Glutathione-(glycine- ¹³ C ₂ , ¹⁵ N) trifluoroacetate		1.9	[M-H] ⁻	25	309.2 → 146.2	15	–	–
Oxidized L-glutathione	GSSG	2.4	[M-H] ⁻	35	611.3 → 306.4	22	n.a.	0.02

n.a. not assessed; LOD, limit of detection; LOQ, limit of quantification; RT, retention time.

Just after sampling, perilymph samples were treated to prevent NAC and GSH degradation as follows. A 10 μL aliquot of a mixture containing N-acetyl-L-(cysteine-¹³C₃, ¹⁵N) at 15 pmol/μL and glutathione-(glycine-¹³C₂, ¹⁵N) trifluoroacetate at 6 pmol/μL in mobile phase was added to 2 μL of perilymph, calibration curve or quality control samples. Proteins were precipitated using 5 μL of 10% trichloroacetic acid. It acidified the pH, which was crucial to stabilize NAC and GSH. Samples were centrifuged for 10 min at 12,000 g, then 10 μL of supernatant was mixed with 10 μL of mobile phase. Every step was performed on ice, and samples were kept at –80 °C under an inert atmosphere (argon).

The injected volume in LC/MS was 5 μL and each sample was measured in triplicate. Calibration curves with 10 points (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 20 ng/μL) were prepared each day before sample analysis. For GSH, the calibration curve was calculated as the sum of the response obtained for its two adducts. Linearity was assessed for NAC (0.6–123 pmol/μL), DiNAC (0.03–62 pmol/μL), GSH (0.33–65 pmol/μL) and GSSG (0.02–33 pmol/μL) with no significant deviation from linearity (runs test, p-value > 0.1). Detection and quantification limits were determined using the signal/noise ratio, respectively, signal/noise > 3 and signal/noise > 10 (Table 1).

2.8.7. Statistical analysis

475 GraphPad Prism software v.7 (GraphPad Software, San Diego, CA, USA) was used for
statistical analysis. Hearing thresholds were compared using two-way analysis of variance
(Anova) with Tukey's post-test. NAC, DiNAC, GSH and GSSG concentrations in perilymph
were analyzed with the non-parametric Kruskal–Wallis test and Dunn's post-test. These
values were expressed as means \pm standard error of the mean. A p-value < 0.05 was
480 considered significant.

3. Results and discussion

The first aim of this study was to develop a formulation of N-acetyl-L-cysteine (NAC), a
precursor of glutathione (GSH), meeting the criteria for transtympanic injection. Then, the
stability and release of NAC from this formulation were investigated. The second objective
485 was to administer HA-NAC-Lip gel in a guinea pig cochlear implantation model in order to
study whether NAC would prevent the damage induced by implantation. The effect on
hearing was assessed both on implanted and non-implanted animals. Correlations between
hearing thresholds and the concentrations of NAC, GSH and their degradation products in the
perilymph were sought after HA-NAC-Lip gel administration.

3.1. Optimization of NAC solutions and liposome formulations

490 As with many water-soluble antioxidants, NAC and GSH are highly sensitive to oxygen,
light, temperature, heavy metals and $\text{pH} > 4$ (Yamamoto and Ishihara, 1994; European
Pharmacopoeia, 2020). Consequently, their stabilization, quantification in biological medium
or encapsulation in nanocarriers are huge challenges (Giustarini et al., 2016; Caritá et al.,
2020). Two entities of NAC oxidize in one DiNAC molecule (Fig. 3), which has no
antioxidant activity and does not regenerate into NAC (Atkuri et al., 2007).

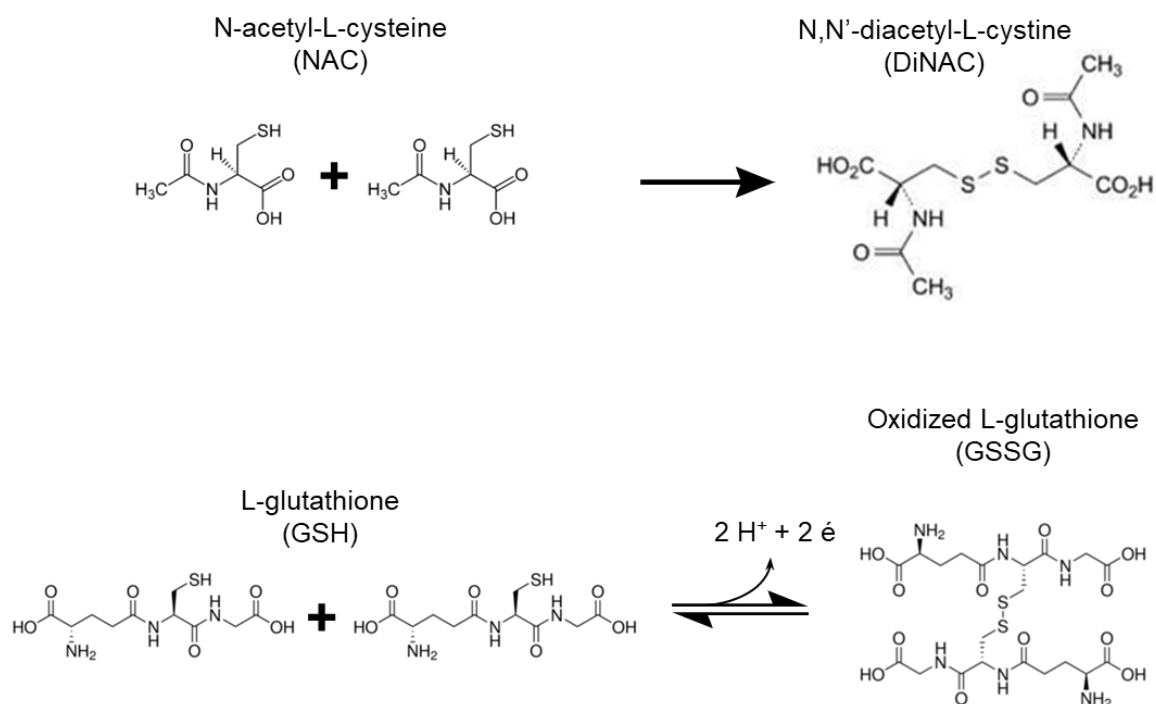


Fig. 3. Oxidoreduction reactions of NAC and GSH in aqueous media

NAC is stabilized by sodium edetate (Anaizi et al., 1997) but this preservative scavenges
 500 Mg²⁺ and Ca²⁺, ions which are essential for inner ear sensory epithelium activity (Nyberg et al., 2019). Furthermore, preservatives are not allowed in transtympanic formulations (Jaudoin et al., 2021).

Without any protection from oxygen and light, NAC in solution at pH 7.4 was rapidly and practically degraded in less than 2 days when increasing the temperature from 4 to 37 °C
 505 (*data not shown*). For these reasons, precautions were taken to limit NAC degradation (*see section 2 Materials and methods*). After argon bubbling in NAC solution for at least 40 min, dissolved oxygen was below 1 mg/mL. NAC was then stable over a 1-month period under storage conditions (4 °C, argon) (10% degradation, 30 g/L, n = 3) whereas 23% of NAC was degraded over this period without argon on top of the solution. Initial NAC solution
 510 protection slowed the degradation kinetics of this antioxidant.

The second issue with the NAC formulation was to obtain a sustained release of intact NAC. In an earlier study, the HA liposomal gel was formulated with EPC-based PEGylated liposomes to sustain the release of dexamethasone phosphate (M = 512.4 g/mol) (El Kechai et al., 2016). These EPC-based PEGylated liposomes migrated from the middle ear to
 515 accumulate in the round window membrane and released their content in a sustained manner in the inner ear fluid. Based on these interesting results, we initially selected this formulation to encapsulate NAC. As NAC is a small hydrophilic molecule (163.2 g/mol), its release from

EPC-based liposomes was almost immediate (*data not shown*). DPPC was reported in liposome formulations containing NAC (Hoesel et al., 2008; Mitsopoulos and Suntutres, 2011; Alipour et al., 2013). This saturated lipid with a transition temperature of 41 °C was chosen to strengthen the lipid bilayer of liposomes and decrease lipid oxidation. To check that NAC was not degraded during lipid film hydration and extrusion of NAC-Lip at 50 °C, a 30 g/L NAC solution whose oxygen was removed, was heated for 2 h at 50 °C under nitrogen. NAC was not degraded (*data not shown*), thus the DPPC-based formulation was selected for NAC encapsulation.

3.2. Liposome characterization

NAC-loaded liposomes (NAC-Lip) showed a monodisperse size distribution (PDI < 0.1) centered around 150 nm (Table 2).

Table 2. Physicochemical and drug loading properties of NAC-Lip (DPPC:Chol:DSPE-PEG₂₀₀₀ 60:35:5 mol%, n = 3)

D _h (nm)	PdI	ζ potential (mV)	[Lipids] (mM)	DL%	EE%	Osmolality (mOsmol/kg)	NAC degradation (%)	[NAC] (g/L)
153 ± 19	0.06	-24 ± 2	74 ± 7	6 ± 1	13 ± 2	284 ± 10	4 ± 1	4.6 ± 2.0

D_h, hydrodynamic diameter; PdI, polydispersity index; DL, drug loading; EE, encapsulation efficiency; NAC, N-acetyl-L-cysteine. Values are means ± standard deviation.

The ζ potential was around -25 mV (Table 2). Unloaded liposomes (Lip) exhibited the same size distribution and ζ potential value, indicating that NAC did not modify the surface of liposomes. The mean drug loading for NAC-Lip was 60 ± 11 mg NAC/g lipids (equivalent to 24 ± 3 mol%/mol) (n = 3). Only a small amount of the degradation product DiNAC was present in the formulation after manufacturing (4 ± 1%).

3.3. NAC stability and release from liposomes under storage conditions

Despite there being many publications on NAC-loaded liposomes (Hoesel et al., 2008; Mukherjee et al., 2009; Alipour et al., 2013; Hamedinasab et al., 2020), DiNAC, the oxidized product of NAC, has never been quantified. Given the instability of NAC in solution, it seemed important to check NAC stability when encapsulated in NAC-Lip before *in vivo* administration. After purification, NAC stability within liposomes (Fig. 4A) and NAC release from NAC-Lip (Fig. 4B) were assessed under storage conditions for up to 1 week (4 °C, argon, dissolved oxygen < 1 mg/mL).

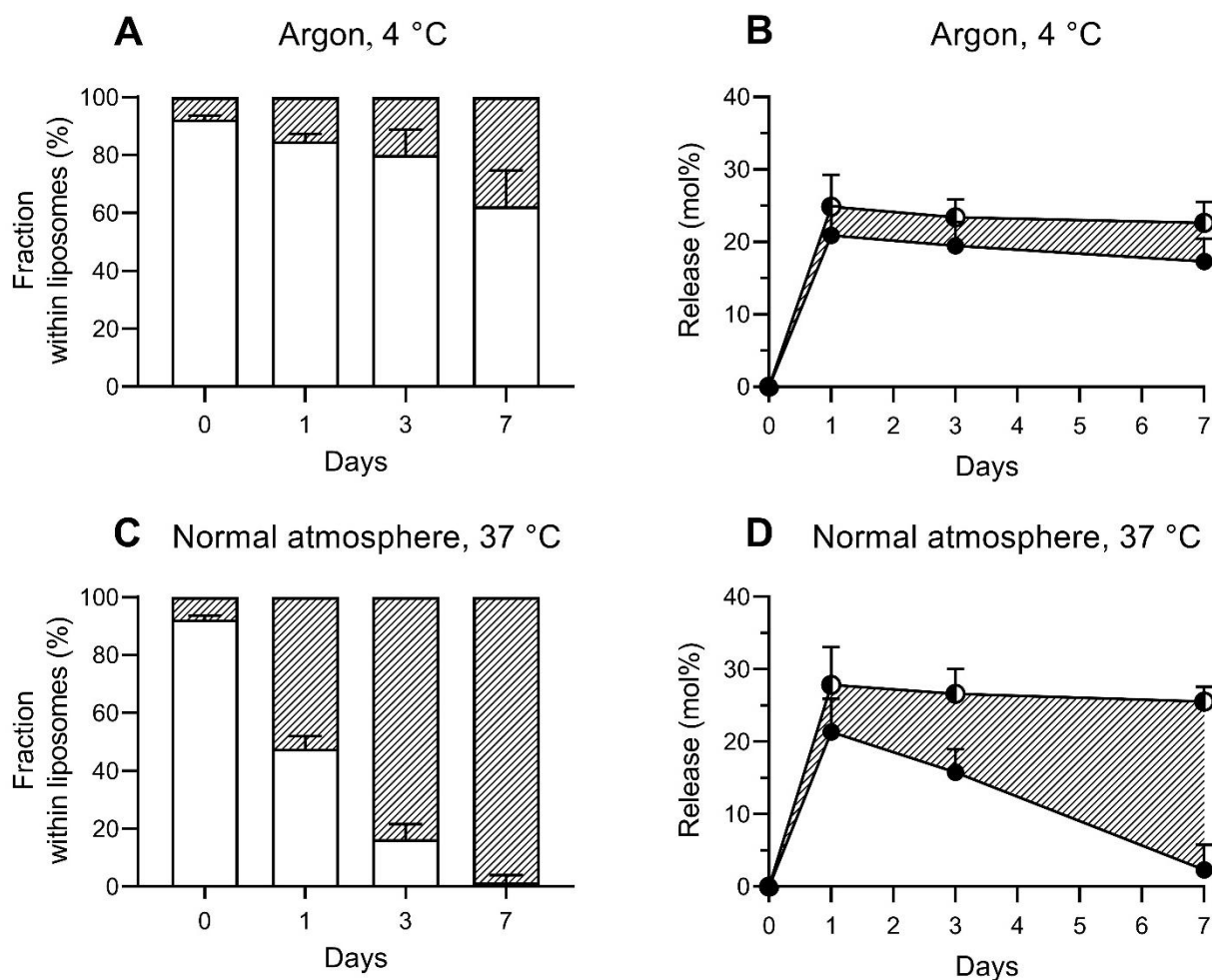


Fig. 4. NAC stability within liposomes expressed as a fraction within liposomes (%) (*white bars*) (A, C) and release of NAC and DiNAC from liposomes (B, D) for up to 1 week. They were evaluated under storage conditions (4 °C, under argon) (A, B) and *in vivo*-mimicking conditions (37 °C, under normal atmosphere) (C, D). NAC (●) and NAC equivalent (NAC + 2×DiNAC) (○) are expressed in mol% on graphs B and D. The shaded area helps to visualize the difference between NAC and NAC + 2×DiNAC which is the fraction of NAC oxidized to DiNAC. Values are means ± standard deviation (n = 3).

Under storage conditions, NAC was predominantly intact within the liposomes (Fig. 4A). However, NAC degradation occurred over time and at D₇, intact NAC reached 62 ± 12% (n = 3). The release of NAC was around 20% after 1 day and then remained stable over a 1-week period (Fig. 4B). Mainly intact NAC was released which is consistent with its stability within the liposomes.

3.4. HA-liposomal gel characterization

560 To minimize the amount of oxidized product (DiNAC) injected *in vivo*, NAC-Lip were systematically prepared 1 day before *in vivo* administration. Thus, $15 \pm 3\%$ of oxidized NAC (n = 3) was present within the liposomes in the formulation injected *in vivo*. The final dose of intact NAC encapsulated within liposomes and administered to the guinea pig was 0.25 ± 0.07 mg (n = 30 ears) and 0.30 ± 0.06 mg (n = 30 ears) for, respectively 113 ± 31 μ L at 565 D₋₃ and 138 ± 28 μ L at D₀ of injected gel. These values were in the range of doses reported in earlier studies of intratympanic administration of solutions or sponges in contact with the round window for only 30 minutes (0.2 to 30 mg) (Choe et al., 2004; Eastwood et al., 2010b; Nader et al., 2010; Saliba et al., 2010; Mohan et al., 2014).

3.4.1. Osmolality and viscosity

570 To avoid perilymph leakage through the round window membrane, formulations intended for inner ear delivery must meet the requirements for intratympanic administration: physiological pH (~7.4) and iso-osmolality with the perilymph (~300 mOsm/kg) (El Kechai et al., 2015a). HA-liposomal gels exhibited these characteristics (Table 3).

Table 3. Osmolality, pH and zero-shear viscosity of the different HA liposomal gels

Gel	pH	Osmolality (mOsm/kg) (n = 3)	η_0 (10^2 Pa.s) (n = 3)
HA-EPC-based PEGylated liposomes	7.4	304 ± 20	5.4 ± 1.0
HA-Lip	7.4	300 ± 10	5.3 ± 1.0
HA-NAC-Lip	7.4	298 ± 15	5.1 ± 1.2

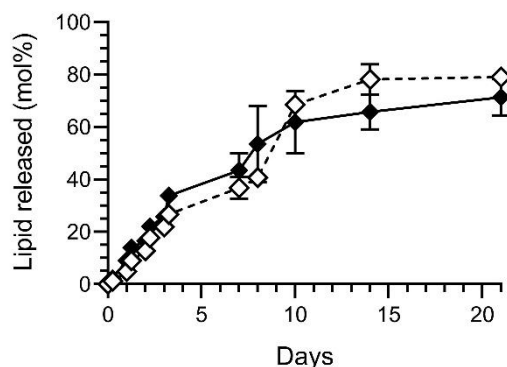
575 η_0 , zero-shear viscosity.

The zero-shear viscosities of HA-Lip and HA-NAC-Lip gels were similar (Table 3). HA liposomal gels retained the shear-thinning and non-thixotropic properties described previously with EPC-based liposomes (El Kechai et al., 2015b).

3.4.2. Release of liposomes from HA liposomal gels

580 The gel microstructure allowed liposome migration into HA gels depending on their surface properties (El Kechai et al., 2017). The migration into HA gels was most significant with EPC:Chol:DSPE-PEG₂₀₀₀ liposomes (PEGylated > anionic > neutral > cationic). Thus, changes in the composition of liposomes (DPPC instead of EPC) might have an influence on their migration properties. As the drug kinetics in the perilymph is likely to rely on the ability 585 of PEGylated liposomes to migrate within the HA network and accumulate in the round

window membrane (El Kechai et al., 2016), this parameter was important to check. Thus, the release of liposomes from HA liposomal gels was evaluated on Transwell® inserts (Fig. 5).



590 **Fig. 5.** Release of Lip (◆) and EPC-based liposomes (◇) from HA liposomal gels over time, on Transwell® inserts at 37 °C (n = 3).

The same release profiles and percentage of lipid released were observed for both liposome formulations. 50% of the initial lipid content in HA liposomal gel was released around D₈. These results confirmed the hypothesis that DPPC-based PEGylated liposomes could be released from HA liposomal gel as previously observed with EPC-based liposomes (El
595 Kechai et al., 2017).

3.5. *In vivo* study

3.5.1. *Effect on hearing function*

Hearing thresholds (dB) correspond to the minimum intensity for which one or several ABR waves are recognized. They were measured at 4, 8, 16 and 32 kHz frequencies and with
600 clicks, corresponding to a set of 2 kHz-centered low frequencies. Frequencies are tonotopically organized throughout the cochlea, with low frequencies in the apical region of the cochlea and high frequencies in the basal region, near the round window membrane and the site of implantation. Normal ABR thresholds were recorded before implantation for all conditions tested (Fig. 6 for preoperative ABR).

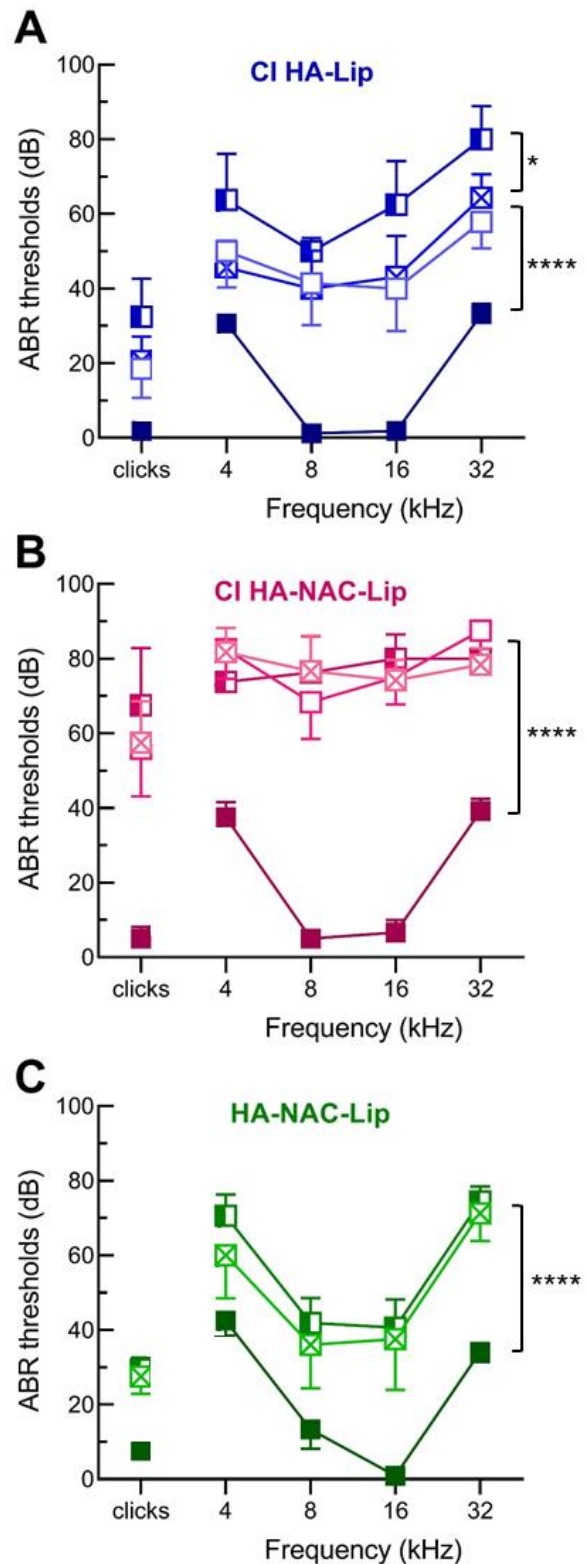
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Fig. 6. ABR thresholds (dB) recorded before (■) and at D₇ (■), D₁₅ (⊠), and D₃₀ (□) post-implantation without (A) or with treatment (B) and treatment without implantation (C).

(A) Effect of cochlear implantation on hearing function (CI HA-Lip, n = 8).

(B) Effect of HA-NAC-Lip on cochlear implantation trauma (CI HA-NAC-Lip, n = 6).

(C) Effect of HA-NAC-Lip gel administration on normal-hearing guinea pigs without cochlear implantation (HA-NAC-Lip, n = 16). Values are means ± standard error of the mean. *: p < 0.05, *****: p < 0.0001 (two-way Anova, Tukey's post-test).



In the CI HA-Lip control group, cochlear implantation induced a severe hearing loss at D₇ (Table 4).

Table 4. Hearing loss at D₇ and D₁₅ compared to preoperative ABR in the CI HA-Lip, CI HA-NAC-Lip and HA-NAC-Lip groups. Values are means ± standard error of the mean

Frequency	Time	Hearing loss (dB)		
		CI HA-Lip group (n = 8 ears)	CI HA-NAC-Lip group (n = 6 ears)	HA-NAC-Lip group (n = 16)
Clicks	D ₇	31 ± 11	65 ± 16	23 ± 3
	D ₁₅	19 ± 9	51 ± 13	19 ± 4
4 kHz	D ₇	36 ± 12	41 ± 8	18 ± 11
	D ₁₅	15 ± 9	45 ± 6	24 ± 8
8 kHz	D ₇	50 ± 4	73 ± 9	22 ± 8
	D ₁₅	39 ± 14	63 ± 10	30 ± 11
16 kHz	D ₇	61 ± 12	78 ± 6	38 ± 8
	D ₁₅	41 ± 16	68 ± 7	38 ± 14
32 kHz	D ₇	48 ± 12	44 ± 4	34 ± 9
	D ₁₅	31 ± 6	40 ± 13	40 ± 13

610 *: p < 0.05, **: p < 0.01 (one-way Anova, Tukey's post-test). ABR: auditory brainstem response.

Auditory function was partly recovered at D₁₅ and remained stable between D₁₅ and D₃₀ (Fig. 6A). At D₁₅, the trauma was more pronounced at 8 and 16 kHz compared to clicks and 4 kHz. This electrode array-induced trauma was similar to that of other studies in guinea pigs (Eastwood et al., 2010a; Mabelle et al., 2017). An earlier study by our group showed the safety of HA liposomal gels on hearing function in guinea pigs after transtympanic injection (El Kechai et al., 2016). No sign of inflammation was observed in the middle ear during the period of the present experiment.

In the CI HA-NAC-Lip group (Fig. 6B), local treatment with HA-NAC-Lip gel dramatically increased the hearing thresholds at all frequencies at D₇. In addition to the cochlear implantation trauma, NAC administration generated pronounced damage when comparing the two implanted groups, with and without NAC at D₁₅ (Table 4). The trauma induced in this group was significantly higher than that observed in non-treated cochlear implanted animals at D₁₅. No recovery was observed between D₁₅ and D₃₀. The adverse auditory effects concerned all frequencies, including clicks that scan a set of frequencies centered around 2 kHz located in the apical part of the cochlea. This clear deleterious effect of NAC when locally administered by transtympanic injection was not expected, given the good tolerance of the drug by oral or intravenous routes (Atkuri et al., 2007).

To better understand these results, HA-NAC-Lip gel was locally injected to normal-hearing not-implanted guinea pigs. Despite preclinical studies on the therapeutic potential of NAC in inner ear applications (Choe et al., 2004; Eastwood et al., 2010b; Nader et al., 2010; Saliba et al., 2010; Mohan et al., 2014), its tolerance on the hearing function has never been reported in guinea pigs. HA-NAC-Lip gel induced significant damage in normal-hearing guinea pigs, even at low frequencies (Fig. 6C). This hearing loss was constant at all of the frequencies between D₇ and D₁₅ (ns, two-way Anova) (Table 4). Thus, the loss was not recovered at D₁₅. The total dose of NAC (0.55 mg) seemed to provoke an irreversible hearing loss in the period studied. Therefore, it was necessary to establish whether the ototoxicity we observed was due to an excessive amount of NAC (or its degradation product DiNAC) in the perilymph or to an overstimulation of glutathione synthesis by the cochlear tissue.

3.5.2. NAC, DiNAC, GSH and GSSG quantification in the perilymph

NAC, DiNAC, GSH and GSSG concentrations were measured in the perilymph over 15 days after HA-NAC-Lip gel injection in non-implanted normal-hearing guinea pigs. Indeed, the perilymph sampling procedure required animal euthanasia, making quantification over time impossible in the CI HA-NAC-Lip group. Drug quantification in the perilymph is very delicate due to the small volume sampled ($\leq 2 \mu\text{L}$). Thus, it is essential to use highly sensitive analytical techniques such as LC/MS. Furthermore, accurate determination of the concentrations of NAC and GSH in biological fluids is a major technical and analytical challenge (Giustarini et al., 2016), because these molecules are rapidly degraded. Two entities of NAC oxidize into one DiNAC molecule, and two GSH entities oxidize into one GSSG molecule, through the formation of a disulfide bond. Stabilizers are commonly used in quantification methods. However, they only allow a total concentration in NAC equivalent ($[\text{NAC}] + 2 \times [\text{DiNAC}]$) or in GSH equivalent ($[\text{GSH}] + 2 \times [\text{GSSG}]$) to be determined (Fig. 3). In the present study, it was relevant to quantify both oxidized and non-oxidized products.

– Release of NAC and DiNAC in the perilymph

NAC was detected in the perilymph at an average concentration of $\sim 0.8 \text{ pmol}/\mu\text{L}$ over 15 days (Fig. 7A).

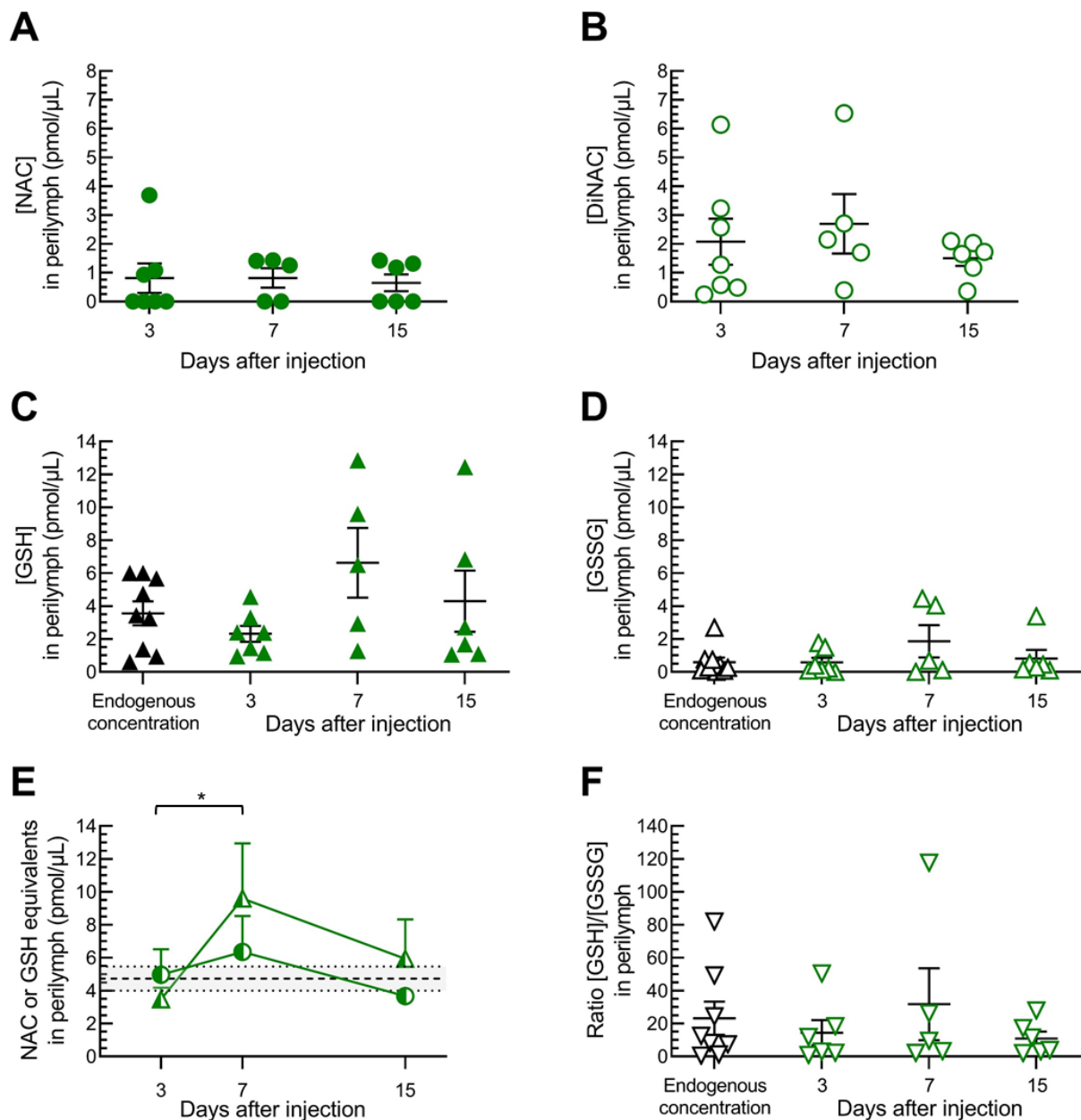


Fig. 7. NAC, DiNAC, GSH and GSSG concentrations in the perilymph over time after transtympanic injection of HA-NAC-Lip gel (HA-NAC-Lip group) without cochlear implantation. (A) [NAC] (●), (B) [DiNAC] (○), (C) [GSH] (▲) and (D) [GSSG] (△) monitored over 15 days in perilymph (pmol/μL) and compared to endogenous concentrations for C and D. (E) NAC equivalent ([NAC] + 2×[DiNAC]) (●) or GSH equivalent ([GSH] + 2×[GSSG]) (▲) (pmol/μL) over 15 days in perilymph for the HA-NAC-Lip group, compared to endogenous GSH equivalent concentration (*hatched line, limits of error bars in dots*). (F) [GSH]/[GSSG] ratio (▽) monitored over 15 days in perilymph (pmol/μL), compared to endogenous concentrations. No significant difference was observed after NAC injection (Kruskal–Wallis, Dunn’s post-test) apart from GSH equivalent concentrations that were different between D₃ and D₇ (*: p < 0.05). Limits of quantification of NAC, DiNAC, GSH and GSSG were, respectively 1.2, 0.06, 1.6 and 0.02 pmol/μL. Values are means ± standard error of the

mean, n = 5–9.

670 Thus, HA-NAC-Lip gel was able to deliver NAC into the inner ear and to prolong its
release. This is in line with previous results obtained with a corticoid (El Kechai et al., 2016)
and is consistent with the presence of the formulation in the middle ear for all of the animals
at D₇. Yet, DiNAC, the degradation product of NAC, was quantified at twice the
675 concentration of NAC in the perilymph (~2 pmol/μL) (Fig. 7B). Therefore, 80% of NAC was
present in its oxidized form in the perilymph. At least ~0.0003% of the total injected dose of
NAC was able to diffuse from the middle to the inner ear at D₃, in its intact (NAC) and
oxidized form (DiNAC).

Before crossing the round window membrane to reach the cochlear fluids, NAC might
have been degraded inside the formulation once injected into the middle ear. To answer this
680 question, NAC stability within liposomes and its release from liposomes were assessed under
in vivo-mimicking conditions, 37 °C, under a normal atmosphere (Fig. 4C and D). NAC
release at 37 °C (Fig. 4D) and 4 °C (Fig. 4B) was equivalent, which was expected regarding
the high transition temperature of DPPC (41 °C). HA-NAC-Lip gel was injected twice at 3-
day intervals, thus D₃ of the pharmacokinetics corresponded to 6 days and 3 days after the
685 first and second administrations, respectively. *In vitro* (Fig. 4C), less than 16% of the initial
dose of NAC remained intact within the liposomes at D₃ and even less at D₆ (n = 3).
Therefore, *in vivo*, a large part of DiNAC assayed in the perilymph had already been
generated before diffusing into the inner ear. The manufacturing process of liposomes
involved many steps. Furthermore, contact between NAC and metals could not be avoided
690 inside the extruder (inox). Despite all of the precautions taken, our results showed that oxygen
had to be totally removed from formulations, which was difficult to achieve at a bench scale.
Nevertheless, the perilymphatic concentrations of NAC and DiNAC remained constant over
time, suggesting that the HA-NAC-Lip system continued to release intact NAC in the
perilymph. The hearing loss observed in the HA-NAC-Lip group might be attributed to the
695 presence of the degradation product, DiNAC.

– *GSH and GSSG concentrations in the perilymph*

To assess the potential stimulation of GSH synthesis by NAC, the endogenous
concentrations of GSH and its degradation product (GSSG) were quantified in the perilymph
of guinea pigs: 3.6 ± 0.7 pmol/μL (Fig. 7C) and 0.7 ± 0.3 pmol/μL (Fig. 7D), respectively

700 (n = 9). To the best of our knowledge, this is the first time that GSH and GSSG concentrations have been quantified in the perilymph.

We did not observe any increase in GSH (Fig. 7C) or GSSG (Fig. 7D) in the perilymph within the time window investigated in the HA-NAC-Lip group. However, a slight increase in total glutathione concentration ($[GSH] + 2 \times [GSSG]$) was noted between D₃ and D₇ (Fig. 7E). 705 Nevertheless, the $[GSH]/[GSSG]$ ratio was unchanged compared to the endogenous ratio (Fig. 7F). One must consider that these concentrations might have been more important in the perilymph outside the time observation window and more particularly before D₃.

3.5.3. Potential of NAC for the treatment of inner ear disorders

As stated in the Introduction (*section 1*), in most previous preclinical and clinical studies, 710 NAC solutions did not meet the specifications for transtympanic administration. Therefore in humans, transtympanic injection of a hyperosmolar solution of NAC (200 mg/mL) led to an acute pain in the ear (Riga et al., 2013). Although we developed an iso-osmolar, neutral-pH formulation for NAC, with no preservatives, the local administrations of NAC were ototoxic in the current study, in both implanted and non-implanted groups. As mentioned previously, 715 unloaded HA liposomal gel proved its safety after intratympanic administration (El Kechai et al., 2016). Consequently, the adverse effects could only be caused by the drug, NAC, or its degradation product, DiNAC.

These results were unexpected, since preclinical and clinical studies showed rather positive effects on audition for other applications when NAC was orally administered (for review, see 720 Kopke et al. (2007)). NAC did not induce any adverse effects on hair cell lines (Feghali et al., 2001; Low et al., 2008), organ of Corti's explants (Maniu et al., 2011) or cochlea explants (Lopez-Gonzalez et al., 1999; Eshraghi et al., 2016). *In vitro* models of the inner ear consider neither the tissue and anatomy complexity of the cochlea, nor the physiological function in presence of acoustic stimulation. Experimental conditions are often different (dose, exposure 725 time, ...). These differences could explain why *in vitro* results are not always predictive of *in vivo* ones. NAC is also a collagenase inhibitor, by chelation of zinc, its cofactor (Berman, 1975). Collagenases are found in the stria vascularis of the cochlea (Wu et al., 2017). A disbalance of their activity can impact the growth of neurites (Sung et al., 2014) and the auditory function (Wu et al., 2017). In another work in guinea pigs, an osteo-densification 730 was noticed around the electrode array after the local administration of NAC (Eastwood et al.,

2010c). Other studies also discussed the capacity of NAC to modulate the osteoblast differentiation and stimulate osteogenesis (Yamada et al., 2013).

The degradation product, DiNAC, was poorly investigated in the literature because this molecule has no therapeutic activity. To the best of our knowledge, in otology, the effects of DiNAC are not documented. DiNAC is a potential modulator of delayed hypersensitivity reactions in rodents with immunomodulatory effects opposite to those of NAC (Särnstrand et al., 1999). However, in absence of cochlear implantation, no foreign body is present in the inner ear to initiate this mechanism. Besides, antiatherogenic and vasodilator effects of DiNAC were reported, likely by NO release (Pettersson et al., 2008).

Constant concentrations of NAC and DiNAC (~ 0.8 pmol/ μ L and ~ 2 pmol/ μ L, respectively) were observed in the perilymph between D₃ and D₁₅. However, we cannot exclude the possibility that higher amounts would be obtained at shorter time scales. Despite all precautions (slow aspiration, volume sampled inferior to 2 μ L), contamination by cerebrospinal fluid cannot be excluded which could underestimate their concentrations (Salt et al., 2003). Indeed, in the recent study by Gausterer et al. (2020), a peak of NAC was achieved very rapidly (~ 214 pmol/ μ L at 3 h) in the perilymph after transtympanic injection of a poloxamer gel (initial NAC dose 2 mg) in guinea pigs. This is the only other work reporting NAC quantification in the perilymph. However, no hearing assessment was performed, so the potential toxicity of NAC at this high concentration is unknown. This rapid passage was not expected from a small hydrophilic molecule since the round window membrane, the main drug access route to the perilymph, is more permeable to lipophilic molecules compared to hydrophilic ones (Jaudoin et al., 2021). NAC entrance into the inner ear might have been favored by its mucolytic properties. In guinea pigs, the whole cochlea protrudes into the middle ear and the otic capsule is thinner at the apex which could favor the transport of NAC. To the best of our knowledge, this point is not documented in the literature for this molecule. For gentamicin sulfate, another hydrophilic drug, Mikulec et al. (2009) estimated that the transport through the apex represented 20–30% of the total drug distribution in the guinea pig cochlea when the bulla is filled. This could explain why, in our study, the hearing loss also impacted low frequencies, in both implanted and non-implanted NAC-treated guinea pigs (Fig. 6). Low frequencies are located far from the round window membrane. However, the passage through the otic capsule is unlikely to occur in humans since the bone is thicker than that of guinea pigs and the cochlea does not protrude in the middle ear cavity like in rodents. NAC diffusion to the last turn of the cochlea is very unlikely, especially without cochlear

765 implantation to drag the drug. For comparison, round window application of gentamicin sulfate did not reach significant concentrations at the apex in guinea pigs (Plontke et al., 2007). The observed toxicity might also be due to the oxidized product, DiNAC, detected in the inner ear fluid at higher concentrations than NAC. It was already present in the injected formulation and could be delivered to the inner ear by crossing the round window and/or the otic capsule. DiNAC might also have been produced after NAC reaction with reactive oxygen 770 species in the perilymph, which is inherent to NAC activity. Although it cannot be ruled out that the adverse effects on hearing might be specific to rodents or that NAC pharmacokinetics may not be transferred to the human situation, NAC does not seem to be a good candidate for local inner ear application in the conditions used in the present study (formulation, number of administration, dose). Indeed, it is possible that the total dose of NAC administered locally 775 was too high (0.55 mg) or that the NAC administration 3 days before trauma induction had a pro-oxidant effect. Some antioxidants might, under certain conditions, have pro-oxidant effects in absence of reactive oxygen species (Aldini et al., 2018) or once in their oxidized form (Prasad and Bondy, 2020).

4. Conclusion

780 Although HA-NAC-Lip gel formulation complied with the criteria for transtympanic injection and sustained the release of NAC in the perilymph over 15 days, it induced an important hearing loss in a cochlear implant model and in normal-hearing guinea pigs. Our study demonstrated that NAC administration (total dose 0.55 mg in 2 injections) is not a relevant approach to prevent damage induced by cochlear implantation. Indeed, the poor 785 stability of NAC under physiological conditions constitutes a major hurdle as its degradation product, DiNAC, was generated in the perilymph at twice the concentration of NAC. Thus, adverse effects on hearing might be attributable to DiNAC. Endogenous concentrations of GSH and GSSG were quantified in the perilymph for the first time. However, HA-NAC-Lip gel did not tend to stimulate their synthesis sufficiently to induce an increase in their 790 perilymph concentration in the time window investigated. Replacement of NAC by another antioxidant might be considered. α -lipoic acid (Jung et al., 2021) and edaravone (Gao et al., 2015) seem promising as they protected the auditory function after local administration in noise-induced hearing loss or drug ototoxicity. Other thiol-containing antioxidants showed promising results for hearing protection such as GSH (Campbell et al., 2003), sodium thiosulfate (Berglin et al., 2011) and D-methionine (Alagic et al., 2011). The main challenge 795 of antioxidants remains the preservation of their stability both in the formulation and *in vivo*.

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Declaration of interest

The authors declare no conflicts of interest.

Sample CRediT author statement

810 **Céline JAUDOIN:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Fabienne CARRE:** Conceptualization, Methodology, Investigation. **Maria GEHRKE:** Conceptualization, Methodology. **Audrey SOGALDI:** Methodology, Validation. **Vincent STEINMETZ:** Methodology, Investigation. **Nathalie HUE:** Methodology, Writing – review & editing. **Catherine CAILLEAU:** Conceptualization, Methodology, Investigation. **Guillaume TOURREL:** Resources, Writing – review & editing. **Yann NGUYEN:** Writing – review & editing. **Evelyne FERRARY:** Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition, Project administration. **Florence AGNELY:** Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition, Project administration. **Amélie BOCHOT:** Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition, Project administration.

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