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Research Paper

Inner ear drug delivery through a cochlear implant: Pharmacokinetics in a Macaque experimental model

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ABSTRACT

Objectives: The method of drug delivery directly into the cochlea with an implantable pump connected to a CI electrode array ensures long-term delivery and effective dose control, and also provides the possibility to use different drugs. The objective is to develop a model of inner ear pharmacokinetics of an implanted cochlea, with the delivery of FITC-Dextran, in the non-human primate model.

Design: A preclinical cochlear electrode array *(CI Electrode Array HL14DD,* manufactured by Cochlear Ltd.*)* attached to an implantable peristaltic pump filled with FITC-Dextran was implanted unilaterally in a total of 15 Macaca fascicularis (Mf). Three groups were created (5 Mf in each group), according to three different drug delivery times: 2 hours, 24 hours and 7 days. Perilymph (10 samples, 1μL each) was sampled from the apex of the cochlea and measured immediately after extraction with a spectrofluorometer. After scarifying the specimens, x-Rays and histological analysis were performed.

Results: Surgery, sampling and histological analysis were performed successfully in all specimens. FITC-Dextran quantification showed different patterns, depending on the delivery group. In the 2 hours injection experiment, an increase in FITC-Dextran concentrations over the sample collection time was seen, reaching maximum concentration peaks (420-964µM) between samples 5 and 7, decreasing in successive samples, without returning to baseline. The 24-hours and 7-days injection experiments showed even behaviour throughout the 10 samples obtained, reaching a plateau with mean concentrations ranging from 2144 to 2564 µM and from 1409 to 2502µM, respectively. Statistically significant differences between the 2 hours and 24 hours groups ($p = 0.001$) and between the 2 hours and 7 days groups ($p = 0.037$) were observed, while between the 24 hours and 7 days groups no statistical differences were found.

Conclusions: This experimental study shows that a model of drug delivery and pharmacokinetics using an active pump connected to an electrode array is feasible in Mf. An infusion time ranging from 2 to 24 hours is required to reach a maximum concentration peak at the apex. It establishes then an even concentration profile from base to apex that is maintained throughout the infusion time in Mf. Flow mechanisms during injection and during sampling that may explain such findings may involve cochlear aqueduct flow as well as the possible existence of substance exchange from scala tympani to extracellular spaces, such as the modiolar space or the endolymphatic sinus, acting as a substance reservoir to maintain a relatively flat concentration profile from base to apex during sampling.

Leveraging the learnings achieved by experimentation in rodent models, we can move to experiment in non-human primate with the aim of achieving a useful model that provides transferrable data to human pharmacokinetics. Thus, it may broaden clinical and therapeutic approaches to inner ear diseases.

Abbreviations: CA, Cochlear aqueduct; CI, Cochlear implant; CSF, Cerebrospinal fluid; EAC, External auditory canal; EIT, Electrode insertion trauma; Gp, Guinea pig; LSC, Lateral semicircular canal; Mf, Macaca fascicularis; RW, Round window; ST, Scala tympani / Tympanic scala.

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1. Introduction

Cochlear implantation represents an outstanding treatment for severe to profound sensorineural hearing loss *(Birman et al., 2020; Huarte et al., 2017)*. Over the past three decades, advancements in the surgical technique and the design of the electrode have provided less traumatic and lower volume electrode arrays, aiming to achieve better hearing preservation rates. However, insertion of an electrode array invariably produces a distress, a well-known phenomenon in cochlear implantation which may cause damage to the inner ear, and particularly to the spiral ligament, organ of Corti, osseous spiral lamina and neural dendrites. Furthermore, the pro-inflammatory microenvironment within the cochlea following implantation often promotes a robust peri-electrode fibrosis which may reduce the efficiency of the device by increasing electrical impedance and also cause postoperative acoustic loss *(Eshraghi et al., 2003; Ishiyama et al., 2016; Linthicum et al., 1991; O'Leary et al., 2013; Venail et al., 2015)*. Several lines of evidence, using experimental models, implicate the glucocorticoid receptor agonist dexamethasone as a beneficial agent for use in the cochlea. It has been shown to reduce cochlear damage and loss of hearing resulting from exposure to ototoxic drugs such as kanamycin *(Himeno et al., 2002)* and cisplatin *(Daldal et al., 2007)*. Additionally, protection from ischemia/reperfusion-induced damage *(Otake et al., 2009)*, noise exposure *(Takemura et al., 2004)* and TNFα-induced auditory hair cell loss *(Dinh et al., 2008)* has been ascribed to this agent. Importantly, protection of residual hearing from electrode insertion trauma (EIT) has been described for dexamethasone *(Braun et al., 2011; Eshraghi et al., 2007; Eshraghi et al., 2011; James et al., 2007; Vivero et al., 2008)*. Glucocorticoid receptor agonists have been shown to reduce a number of mediators that promote fibrosis and have been proposed to potentially improve the performance of the cochlear implant (CI) through reducing the fibrotic sheath formation that occurs after implantation, thereby minimizing impedances *(De Ceulaer et al., 2003)*.

Therefore, the protective effects of local and systemic administration of corticosteroids during CI surgery have been investigated. Intratympanic administration of a combination of dexamethasone and hyaluronic acid has been reported to allow less traumatic insertion of the electrode and better sealing of the inner ear and to have a positive effect on preservation of hearing in the initial postoperative period *(Ramos et al., 2015)*. However, this approach does not have a sustained therapeutic effect and cannot suppress the secondary chronic inflammation, so a sustained drug administration may be warranted Hendricks et al. (2008) analyzed the different existing techniques for drug delivery that can be integrated into modern auditory prosthetic devices. They conclude that localized, sustained drug delivery directly to the target tissues has several advantages over systemic application, including fewer adverse effects, reduced ototoxicity, smaller quantities of drug used, and better therapeutic outcome. Following these concepts, two main pathways have been developed to deliver drugs into the cochlea in conjunction with a CI. The first is a CI that incorporates the drug into the materials of the electrode array, or into a coating on the electrode array. This technique is believed to be a safe method for cochlear application of corticosteroids *(Farahmand Ghavi et al., 2010; Manrique-Huarte et al., 2020)*, assuring a sustained, effective local dose of the drug *(Hahn et al., 2012)*. However, this approach has some drawbacks such as the variability in dosage control, a predefined fixed term delivery and the significant challenges in implementing different drugs into the system. The second technique that has been developed delivers drug from an implantable pump. The pump is connected to a CI electrode array, which has an in-built cannula. This method of delivery ensures the possibility of long-term delivery and effective dose control, and also provides the possibility to use different drugs. The use of a pump and cannula, while having great potential, also has some potential technical challenges. For example, cannula obstruction may occur at low flow rates, cannula occlusion may occur from bending of the cannula, and repeat transdermal refills indicate that microbiological control is necessary.

A systemic treatment approach for treatment of the inner ear is fraught with difficulties. Achieving adequate dosing to the remote location of the inner ear, which is behind the blood labyrinth barrier often requires high systemic dosing which can lead to multiple side-effects resulting in an unusable therapeutic.

New approaches to treatment of auditory and vestibular pathology aim to preserve the function and structure of the ear *(Brown et al., 2018)*.

It has been shown that the substances applied to the round window or to the perilymph of the inner ear are not distributed uniformly throughout it *(Salt et al., 2015)*. As such it is not possible to assume that the drug distribution in the perilymphatic compartments is homogeneous throughout the entire cochlea. Thus, local administration of drug to the cochlea requires a detailed analysis, not only of the anatomy of the cochlea, but also of the injected substance itself and its behavior once the target structure is reached. Pharmacokinetics is the branch of pharmacology that analyzes this behavior of the substance under study. In this way, an adequate knowledge of cochlear pharmacokinetics and of its basic principles: the LADME scheme ("Liberation, Absorption, Distribution, Metabolism, Elimination") will guide and determine the choice, dosage, time and speed of drug administration, as well as the release strategy and the choice of a correct route of administration when assessing the probable success or benefit of a certain therapy. It will also provide information as to the possible obstacles that could arise during its application.

The pharmacokinetics of the cochlea are poorly understood. The cochlea is a relatively isolated organ in the body, surrounded by bone and the air filled cavity of the middle ear. This structure is separated from blood by the blood labyrinth barrier. This isolation means that systemic delivery of drugs for the treatment of inner ear diseases has been proven to be challenging without, up until now, few successful systemic approaches in clinical trials.

Given the current lack of knowledge and the existing difficulties when it comes to deepening the understanding of treating the inner ear, various studies have been carried out over the last years in order to get to know the most adequate and accurate means for the analysis of cochlear pharmacokinetics. These studies have utilized multiple methods, including direct methods, such as perilymph sampling at different levels of the inner ear, and indirect methods, such as the analysis of distortion product otoacoustic emissions (DPOAE) and auditory brainstem response (ABR), or those carried out through imaging test, analyzing both magnetic resonance imaging and micro-computed tomography *(Moudgalya et al., 2019)*.

The inner ear pharmacokinetic profile of any drug, as is the case with dexamethasone, may vary *(Salt et al., 2015)* depending on the rate of entry through the round window (RW), distribution along scala tympani (ST) by diffusion, volume entry of cerebrospinal fluid (CSF) through the cochlear aqueduct (CA), clearance of drug through exchange with CSF, distribution into adjacent compartments (spiral ligament, spiral ganglion, organ of Corti,...) and clearance to blood via the vasculature of the adjacent tissue spaces.

In the field of inner ear pharmacokinetics, the vast majority of research has taken place in the guinea pig (Gp) *(Mynatt et al., 2006; Salt et al., 2017)*. While it is a useful model to establish the basic physiological principles governing the pharmacokinetics of the cochlea, it is not clear how the inner ear pharmacokinetics in rodents translates to humans.

In 2019, Burton et al. carried out a study based on the analysis of acoustic trauma in nonhuman primates. It described the exist-

ing proximity, including at genomic level, reaching a similarity of 93.5% of the genomic sequence, between these animal species, especially the subspecies belonging to the Old-World monkey's group (such as Macaca rhesus, Macaque fuscata, Macaca nigra or Mf) and humans, as well as the specific factors that would condition the future extrapolation of the results obtained to the implementation of therapeutic strategies in clinical practice. As these authors surmise, and although there are variations between species of non-human primates, the ranges of auditory frequencies are very similar to those of the human. Thus, audible frequencies in humans cover ranges between 20-20000Hz *(Burton et al., 2019; Kirk et al., 2009),* while the results obtained in various studies throughout the history of these frequencies in nonhuman primates are variable. While Kirk et al. (2009) described hearing abilities in Macaca nigra from 0.028 to 38.5 kHz, Dylla et al. (2013) studied a range between 0.25 to 40 kHz in Macaca rhesus. In earlier studies, Heffner et al. (1986) described a bigger spectrum in Macaca fuscata, similar to that one described by Moody et al. (1980), ranging from 0.032 to 64 kHz and 0.063 to 64 kHz, respectively. It should be noted, however, that the highest sensitivity in the U-shaped audiogram is recorded at frequencies between 500-4000Hz and 1000-16000Hz in humans and macaca of the Cercopithecidae (Old World Monkeys) family (analyzed in this case in Macaca rhesus), respectively *(Burton et al., 2019).*

Leveraging the learnings achieved by experimentation in rodent models, we can move to experiment in non-human primates, with the aim of achieving a model that provides transferrable data to human pharmacokinetics, enabling the discovery of more effective drugs, delivery systems, and clinical trials for hearing therapies.

The CI provides the opportunity to deliver a combined therapy of drug and electrical stimulation for restoration of the sense of hearing. The drug may be provided to protect residual hearing or to improve CI outcomes by sustaining neural tissue and by reducing the formation of fibrous tissue between the electrodes and the target neural tissue. Recently, Salt et al. (2017) have demonstrated that pharmacokinetics of the sealed and homeostatic cochlea is acutely different than that of the implanted cochlea. Understanding this change in pharmacokinetic behaviour is critical when trying to design an effective and safe combined drug-CI therapy. It is not clear if this change is rodent-specific or if it could be equally extrapolated to primates.

The objective of this study was to develop a model of inner ear pharmacokinetics in the non-human primate model. This model will then be used to evaluate the inner ear pharmacokinetics of an implanted cochlea, with various model molecules or active pharmaceutical forms.

2. Materials and methods

2.1. Overview

The study consists of formal analysis of drug delivery experiments, using a cochlear array electrode with an implantable peristaltic pump attached, and the subsequent sampling of perilymph in Macaca fascicularis (Mf). For this experiment three groups were created, depending on the three different drug delivery times proposed: 2 hours, 24 hours and seven 7 days, following the same conditions and procedures each time.

Fifteen Mf were divided in 3 groups of five animals each. The first group, named "2-hours group", were implanted unilaterally with the cannulated CI electrode and the pump was run for two hours, delivering FITC-Dextran. Following the two-hour delivery perilymph sampling of the apex of the cochlea was performed. Each macaque was designated Mf1-2h, Mf2-2h and so on. A second group named "24-hours group" of 5 Mf was implanted unilaterally with the cannulated CI electrode and pump,

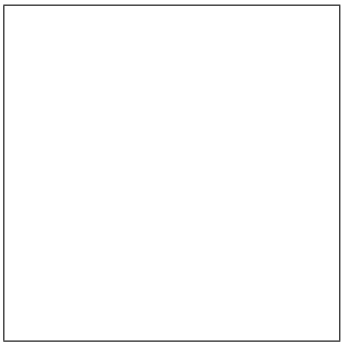

Fig. 1. The implant-cannula-pump system. Fig. 1a: The device used in this study consists of a CI Electrode Array HL14, *Hybrid-L 14 Drug Delivery Variant Animal Array (HL14.DD Electrode Array)* (Cochlear Ltd.), composed of 14 electrodes, with a total length of 10.5mm and a maximum diameter of 0.5mm. It includes a pump, where the FITC-Dextran will be introduced. A silicone tube connects this pump with a stainless coupler that is attached to the cannula outlet of the electrode array. **Fig. 1b:** The incorporated cannula exits at 4mm apical from the white marker. The depth reached in case of full insertion corresponds to 11.5 mm, outlined over the total cochlear length with the animal's tonotopic distribution according to Moody *(Moody et al., 1980).*

delivering FITC-Dextran for 24 hours. After 24 hours delivery time, another surgical procedure was performed to take samples of the apex of the cochlea. According to the findings described by Mynatt et al. (2006), in Gp, each sample obtained would correspond to a different level of the ST. As this work group affirms, when the samples are taken from the cochlear apex, the perilymph extracted from the cochlea is replaced by CSF, entering at the ST through the CA. Thus, the first sample (S1) would represent the concentration of the apex of the cochlea, containing perilymph from the most apical region while later ones would come from the basal turn from increasingly basal regions. Animals in this group were designated: Mf1-24 h, Mf2-24h and so on. Lastly, a group of 5 Mf named "7-days group" were implanted unilaterally with the cannulated CI electrode and pump delivering FITC-Dextran for 7 days. Following 7 days of delivery an apex cochleostomy was performed and samples were taken. The animals in this group were designated Mf1-7d, Mf2-7d and so on. For every specimen, sampled FITC-Dextran was measured immediately after extraction. Macaques were sacrificed, plain x-Rays taken and histological analysis was performed.

2.2. Cochlear drug delivery system and FTIC-dextran

The CI Electrode Array HL14DD is a preclinical research array for intra-cochlea drug delivery manufactured by Cochlear Ltd. It is a modified version of the previously described HL14 array *(Shepherd et al., 2011)*, with the addition of a cannula for fluid sampling or delivery. Specifically, it has 14 electrodes and is 10.5 mm long from the most basal electrode to the distal tip of the array; the tip diameter of the HL14DD array is 0.35 mm, increasing to 0.5 mm at the basal electrode that is located 10.5 mm from the tip of array. *Fig. 1* shows the HL14DD electrode array, including the incorporated cannula which exits 4 mm apical from the white marker. Depicted is also the depth reached in case of full insertion of 11.5 mm. It is outlined over the total cochlear length of one of the animals investigated in this study with the animal's tonotopic distribution according to Moody et al. (1980).

Before the pump was connected to the cannulated electrode array, FITC-Dextran was prepared under sterile conditions. Components were mixed as follows: 10 mM FITC-Dextran (fluorescent dextran, FW -4000, Sigma-Aldrich, St. Louis USA), concentration: 40μg of FITC-Dextran per 1 ml of (MEM α; 51200–038; no phenol red; Fisher Scientific: www.thermofisher.com). Then, approxi-

mately 1.175 ml of FITC-Dextran was filled into the micro infusion pump (iPRECIO SMP-200 ®, Tokyo, Japan), under sterile conditions using a 1 ml syringe with a 27 Gauge cannula. Once the pump was filled and checked for trapped air, the stainless steel coupler (SS coupler) was attached to the cannula outlet of the electrode array *(Salt et al., 2017)*. Delivery at a rate of 2 µL/h was programmed with the iPRECIO software before insertion *(see Fig. 1)*.

2.3. Experimental animals

Animals weighed between 1,850 and 3,550 kg. Specimens were housed at the University of Navarra Animal Facilities and treated in compliance with European Union Regulation 86/609, and in accordance with protocols approved by the Animal Care and Use Committee of the University of Navarra (file number 083/18).

This macaque species (Mf) was chosen for its close phylogenetic proximity to humans that often provides a critical link between basic research and human clinical applications. The surgical technique employed is, therefore, analogous to the one used in humans, as we have already demonstrated *(De Abajo et al., 2017; Manrique-Huarte et al., 2018)*. This fact is of utmost importance to achieve the objectives proposed and to extrapolate results to clinical practice.

2.4. Anaesthesia procedure

All specimens were anesthetized, under analgesia and monitorization following the protocol described for this species *(Panadero et al., 2000)*.

Mechanical immobilization was induced by an intramuscular injection of ketamine (5 mg/kg), midazolam 0.5 mg/kg), and atropine sulfate (0.05 mg/kg). During the surgical procedure, general anaesthesia was induced with Propofol (1 mg/kg); 10 μg of fentanyl were administered IV before the first incision; the animals were maintained with nitric oxide, oxygen 50%, Sevofluorane (2 to 3%), and a fentanyl perfusion during the surgical procedure (2 to 4 mg/kg/hr or Ultiva 0.5 to 0.9 μg/kg/min). Corticosteroids were not systematically administered during postoperative and follow-up period.

2.5. Surgical procedure

The surgical procedure was carried out in two stages: 1) The first stage consisted of the implantation of the device and the activation of the FITC-Dextran release; 2) The second stage was created an apical cochleostomy and enabled the sampling of perilymph, for subsequent analysis. The time elapsed between these two steps depended on the experimental group: 2 h, 24 h or 7 days.

1. First stage: Device implantation. A classic retroauricular incision was made, without extending superiorly, away from the retroauricular sulcus (approximately 2 cm). The muscular plane was exposed, being able to clearly differentiate a line coincident with the temporal line and that divides the temporal muscle superiorly and the cervical musculature inferiorly. Anteriorly, soft tissue was dissected to identify the external auditory canal (EAC) around its complete area, it was exposed, sectioned and sutured. An incision on it was made and the cervical musculature was dissected, exposing by this way mastoid and occipital bone. A subperiosteal and submuscle pocket was made for the pump. After removing EAC cartilage, the osseous segment of EAC, a radical mastoidectomy was performed, respecting tegmen and sigmoid sinus. The Lateral Semicircular Canal (LSC) was identified in depth and the rest of mastoid cells were removed. The posterior and inferior edge of the mastoidectomy was preserved and a tunnel was drilled to fix in that place the SS coupler.

After removing the osseous segment of EAC, malleus and incus were identified. Chorda Tympani was sacrificed. Incus and stapes were disarticulated. Malleus, incus and tympanic membrane were removed. The site for the apex cochleostomy was located between Jacobson nerve, malleus muscle and carotid artery. The RW and its membrane were identified, as well as the stapes. In some cases, we needed to drill the upper edge of the RW niche to have a complete view of the membrane and a proper trajectory of insertion. With a needle *(Ref DN2735B, Terumo Corporation, Tokyo- Japan)* an incision in the RW membrane was made. The size of this incision was adjusted to the diameter of the electrode array to prevent occurrence of perilymphatic fistula. Next the HL14DD electrode array was inserted and secured by sealing the RW with fascia and/or cyanoacrylate *(Permabond 101 ULTRA LOW viscosity, Winchester, Reino Unido)*.

The pump was placed in the previously created pocket in the occipital region. The pump has a tube length of 2.5 cm. The cable of the electrode array was accommodated in the mastoid, and efforts were made to avoid sharp bends in the cannula. Then, the cannula was fixed to the lower margin of the mastoid by means of a stitch at the level of the SS coupler, in order not to interfere with the cannula or the cables.

X-ray analysis was performed to assess depth of insertion of the electrode array.

2. Second stage: Perilymph sampling. Opening of previous wound and removal of fibrous tissue was performed. The site for the apex cochleostomy had been previously located between Jacobson nerve, malleus muscle and carotid artery. The Jacobson's nerve, the tendon of the malleus muscle and the muscle itself were identified. We drilled the bone layer of the apical region of the cochlea with a 1.5 mm diameter drill at speeds below 4000 rpm. The last endosteal layer was preserved. The internal wall of the tympanic cavity was carefully dried. Once this objective had been achieved, following the method described by Salt, A. *(Salt et al., 2006)*, cyanoacrylate was placed. A silicone cup *(Kwik-Cast World Precision Instruments, Sarasota, FL)* was then created. Without interrupting the operation of the pump, a last step of the apical cochleostomy was performed with a pick angle *(Storz 1/3mm 30° House stapes pick N1705 80, Bausch and Lomb inc.)*, removing the remaining endosteum and opening the cochlea. An output of perilymph could then be observed.

A total of 10 samples were taken consecutively using capillary tubes *(Blaubrand® Ref 708707. Wertheim, Germany)*. At least 1μL was taken for each sample. Samples were transferred into dark Eppendorf tubes *(Sartstedt AG& Co. Numbrecht, Germany)* for analysis, thus avoiding altering the fluorescent properties of FITC-Dextran. The pump was removed whilst the cable, cannula and electrode array were left in situ for further histological study. X-ray analysis was performed to check depth of insertion of the electrode array.

2.6. Sampling analysis

Fluorescein concentration was measured in each sample (T1-T10) with a spectrofluorometer *(SpectraMax Gemini XS, Molecular Devices, San Jose, CA, USA)*, using the following settings: 485 nm (excitation), 530 nm (emission) and auto cutoff. A calibration curve was prepared for each experiment, covering a range of FITC-Dextran concentrations (1 µM to 10 mM).

2.7. Temporal bone extraction and histological processing

The petrosal ridges were extracted for histological processing following the classic methodology used in temporal bone laboratories *(Schuknetch (1968)*. The extracted specimens were then dehydrated and embedded in epoxy resin *(Manrique et al., 2014)*. After taking pictures of the block generated by the temporal bone and the resin and maintaining the electrode array in place, the material was sand cut, establishing serial slices every 100 μm, dyed with

toluidine blue, and photographed with a microscope-stereoscope *(Leica ®S8AP)* (magnification 1.25, 1.6, 2, 4, and 6.3fold) for the posterior analysis of histological findings.

2.8. Histological analysis

Histological analysis was performed so as to assess intracochlear location of the electrode array, the presence of any tissue reaction surrounding the electrode array and the cannula (at the level of the RW, the ST and the Vestibule), if any damage at the level of intracochlear structures was observed (spiral ligament, stria vascularis, spiral lamina, cochlear aqueduct), and last, location and measurement of apical cochleostomy. EIT was classified based on Eshraghi classification *(Eshraghi et al., 2003)*.

Furthermore, an estimation of perilymphatic volume was performed using an imaging processing package named FIJI, based on ImageJ. This program allows quantification of such spaces based on histological images. A semi-automatic procedure was applied in order to compute the volume of the different lymphatic spaces within intracochlear structures. Quantification is based on a Macaca fascicularis, not implanted and histologically processed in paraffin *(Manrique-Huarte et al., 2020)*. The approach is the following: for each scala (tympani, vestibuli and media), the user clicks in some point of the cavity. An automatic algorithm puts a seed in that point checking if the pixel exceeds a grayscale threshold. If so, the pixel is segmented with a specific colour. The next step of the algorithm is to apply the same method with its 8 neighbouring pixels. The algorithm will stop when it does not find any neighbouring pixels above the threshold. Then, another algorithm counts the number of each colour pixel in each image. Finally, knowing the pixels dimensions and the number of segmented pixels, the volume of each cavity is calculated by interpolating between 2 images the volume of each segmented region (Fig. 8).

2.9. Statistical analysis

Factorial Mixed ANOVA was performed to test for differences of FITC-Dextran concentrations at each sample between the 2h, 24h and 7days groups. Due to the presence of significant interaction between the samples and groups, LSD contrasts were computed using Z-Scores at first and last samples to check for differences between the groups. Normality assumptions have been checked using Shapiro-Wilk test and Quantile-Quantile plot. Sphericity assumptions have been checked using Mauchly's Sphericity test. A P-value below 0.05 was considered as statistically significant. P values are reported with their corresponding F Statistic and degrees of freedom ($F^{df1;df2}$) or Z-Scores. All statistical analysis was performed using IBM SPSS Statistics V22.

Sample size determination has been carried out using Resource Equation method, which suggests that the degrees of freedom of the error term of the ANOVA should be between 10 and 20. Taking into account that we have 3 groups the sample size should be between 5 and 8 Mf. Due to the 3R principles we utilized 5 animals per group. The 3Rs stand for Replace, Reduce, Refine and represent a responsible approach to animal testing. The goal is to replace animal experiments whenever possible. In addition, the aim is to keep the number of animal experiments as low as possible and to only use the necessary number of animals. Lastly, it is vital to ensure that the distress inflicted upon the animals is as low as possible.

3. Results

These are the first studies carried out in Mf, in which cochlear pharmacokinetics is analyzed by studying the distribution of FITC-Dextran delivered by a cannulated CI electrode placed in ST. Table 1 summarizes the results obtained in this study.

3.1. Surgical findings

Surgery was performed successfully in all 15 Mf. Full RW electrode insertion, without kinking was observed. We had 2 delivery system failures, for different reasons, in Mf1-24h and Mf1-7d.

3.1.1. 2-hours group

Full electrode array insertion (11 mm length) was undertaken through the RW in all 5 Mf. (Length of insertion for each specimen. Mf1-2h: 180-270°; Mf2-2h: >270°; Mf3-2h: 180-270°; Mf4L-2h: 180-270°; Mf5-2h: 180-270°). Sealing was performed with temporal fascia for Mf1-2h and Mf5-2h whereas cyanoacrylate was placed at the level of the membrane for Mf2-2h and a combination of fascia and cyanoacrylate in Mf3-2h and Mf4-2h. During revision surgery, 2 hours after implantation, fluorescein leakage within the middle ear spaces, near to the RW, was perceived for Mf1-2h, Mf2-2h, Mf3-2h, Mf4-2h and Mf5-2h. The apex cochleostomy was performed without complications in all cases and perilymph sampling of a total of 10 samples, was achieved. Time elapsed during sampling varied between specimens: 35 minutes for Mf1-2h, 37 minutes for Mf2-2h, 31 minutes for Mf3-2h, 21 minutes for Mf4-2h and 13 minutes for Mf5-2h.

3.1.2. 24-hours group

Full electrode array insertion (11 mm length) was performed through the RW in all 5 Mf (Length of insertion for each specimen. Mf1-24h: 180-270°; Mf2-24h: 180-270°; Mf3-24h: 180-270°; Mf4-24h: 180-270°; Mf5-24h: 180-270°). The area was sealed with temporal fascia and cyanoacrylate in all cases. After 24 hours revision surgery was performed. No wound dehiscence was seen, and some clots occupied the mastoid area. No RW fluorescein leakage or migration of electrode array was observed in any case. An apex cochleostomy was performed in all cases with no complications. However, outflow of clear perilymph (i.e. potentially with no FTIC-Dextran present) was observed in Mf1-24h. Furthermore, in this animal, the cannula was observed to contain air rather than fluorescein. The time consumed for perilymph sampling was highly variable, and recorded as follows: 24 minutes, 40 minutes, 117 minutes, 108 minutes and 35 minutes for Mf-24h 1, 2, 3, 4 and 5, respectively.

3.1.3. 7-days group

Similar to the 2 hours and 24 hours group, electrode array insertion was complete in all cases (11 mm) (Length of insertion for each specimen. Mf1-7d: >270°; Mf2-7d: 180-270°; Mf3-7d: 180-270°; Mf4-7d: 180-270°; Mf5-7d: 180-270°) and sealed with temporal fascia and cyanoacrylate. No perilymph leakage or migration of electrode array was perceived at revision surgery in any case. An apex cochleostomy was performed in all 5 Mf. At revision surgery, for Mf1-7d, the cannula was detached from the pump at the level of the SS coupler. Perilymph sampling was carried out without event, though it was slow, recorded as follows from Mf1-7d to Mf5-7d: 78, 64, 45, 70 and 76 minutes respectively.

3.2. FITC-dextran quantification

3.2.1. 2-hours group

FITC-Dextran quantification showed a progressive increase in concentration reaching its maximum mean level at S7 and decreasing slightly afterwards. In the Gp, following Salt's methodology *(Salt et al., 2017))* the final samples are expected to consist of primarily CSF with lower drug concentration than the basal perilymph samples. This was not observed in the MF in these experiments. Results are summarized in *Fig. 2*. Mean FITC-Dextran concentration from S1 to S10 was 208 μM, 285 μM, 387 μM, 461 μM, 552 μM, 621 μM, 663 μM, 655 μM, 631 μM, 663 μM, respectively.

Table 1
Summary of the main results obtained by studying the distribution of FITC-Dextran delivered by a cannulated CI electrode placed in ST. Abbreviations. SA: Surgical approach; De: Device; RW: Round Window; RW C: RW Cochleostomy; RW D: RW Drilling; SP: Sampling; EC: Eshraghi classification; EaP: Electrode Array Position; A: Apex; AC: Apex Cochleostomy; HF: Histological Findings; FITC_D: Concentrations; SP: Surgical procedure; PF: Pump failure; CD: Cannula disconnection; F: Fascia; G: Glue (Cyanoacrylate); ST: Scala Tympani; BChip: Bone Chip; S: Sample; SM: Maximum concentration; R: Range; ISF: Inter Scala Fracture; SLF: Spiral Lamina Fracture; ND: No Data.

2-h G	*SA*	*De*	*RW C*	*Sealing*	*RW leak*	*SP*	*EC*	*EAP*	*Length°*	*A C*	*H F*	*FITC_D (μM)*	*Comments*
Mf1 R	OK	OK	RW	F	Yes	10 S 35 min	0	ST	180-270	OK	EA partially out	S1 7 S10 M 458 R 2-458	Device OK SP OK FITC-D OK Upward curve
Mf2 R	OK	OK	RW	G	Yes	10 S 37 min	0	ST	>270	OK	A: BChip	S1 ND S5M 809 R: 436-809	Device OK SP OK FITC_D OK Up-Down curve
Mf3 R	OK	OK	RW	G	Yes	10 S 21 min	0	ST	180-270	OK	EA extracted EA fibrous sheat A: BChip	S1 4 S3M 416 R 4-416	Device OK SP OK FITC_D OK Upward curve
Mf4 L	OK	OK	RW	G+F	Yes	10 S 31 min	1	ST	180-270	OK	RW: G A: BChip and ISF	S1 97 S10M 1177 R 30-1177	Device OH SP OK FITC_D OK Upward curve
Mf5 R	OK	OK	RW	G	Yes	10 S 10 min	1	ST	180-270	OK	A: BChip	S1 936 *3.5μL S1M 936 R 681-936	Device OK SP OK FITC_D S1 increase Flat curve
24h G													
Mf1 R	OK	PF	RW D	G+F+G	No	10 S 24 min	1	ST	180-270	ND	Basal turn: Scar?	S1 2 S9 M 4 R 1-4	Device Failure SP OK FITC-D Low
Mf2 R	OK	OK	RW D	G+F+G	No	10 S 40 min	0	ST	>270	OK	RW: G A: BChip and ISF	S1 2711 S2M 3722 R: 2177-3722	Device OK SP OK FITC_D OK Flat curve
Mf3 R	OK	OK	RW D	F+G	No	10 S 117 min	0	ST	180-270	*2nd turn	RW: G 2nd turn: BChip	S1 2192 S4M 2562 R 2028-2562	Device OK SP OK FITC_D OK Flat curve
Mf4	OK	OK	RW D	F+G	No	10 S 109 min	0	ST	180-270	OK	RW: G Apex: BChip	S1 1981 S20M 2666 R 1981-2666	Device OH SP OK FITC_D OK Flat curve
Mf5 R	OK	OK	RW	F+G	No	10 S 35 min	1	ST	180-270	OK	RW: G	S1 1780 S4M 2020 R 1495-2020	Device OK SP OK FITC_D OK Flat curve
7-d													
Mf1 R	OK	CD	RW D	F+G	No	11 S 78 min	1	ST	>270	OK	A: BChip and SLF and ISF	S1 25 S7 M 27 R 3-27	Device Failure SP OK FITC-D N0
Mf2 R	CSF leak	OK	RW D	F+G	No	10 S 64 min	0	ST	180-270	ND	RW: G	S1 2476 S3M 2584 R: 674-2584	Device OK SP OK FITC_D OK Flat curve
Mf3 R	OK	OK	RW D	F+G	No	13 S 45 min ↓ S5	1	ST	180-270	OK	A: G? 2nd turn; BChip	S1 2026 S1M 2026 R 9-2026	Device OK SP OK FITC_D decline S5 Downward curve
Mf4 R	OK	OK	RW D	F+G	No	10 S 70 min	1	ST	180-270	OK 2nd C BT	RW: G A: SLF BT: BChip	S1 2051 S70M 2313 R 1774-2313	Device OH SP Double C FITC_D OK Flat curve
Mf5 R	OK	OK	RW D	F+G	No	10 S 76 min	0	ST	180-270	OK	A: BChip	S1 3456 S4M 3528 R 2655-3528	Device OK SP OK FITC_D OK Flat curve

Mf5-2h showed higher concentrations than the others in this group ranging between 936 μM and 1777 μM. Note that during S1 perilymph outflow was surprisingly fast and 3μL of analyte was collected. At S10 FITC-Dextran concentration was 756 μM.

3.2.2. 24-hours group

Mean FITC-Dextran concentrations from S1 to S10 were 2166.1 μM for S1, 2532 μM, 2256 μM, 2539 μM, 2564 μM, 2304 μM, 2446 μM, 2299 μM, 2225 μM, 2145 μM, respectively. Given the data, it appears that a plateau is reached at this time point with small rel-

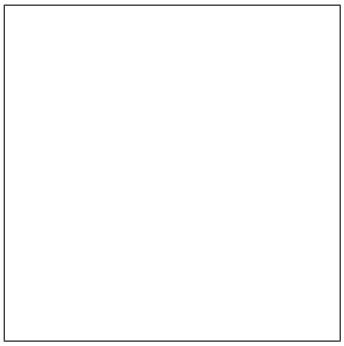

Fig. 2. Sampling results from the 2 h, 24 h and 7 days group. The following graphs summarize the concentrations of FITC-Dextran obtained throughout the 10 samples for each specimen under study, as well as the mean concentrations obtained for each group. The progressive increase in concentrations in the 2 hours group can be appreciated, as well as the maintenance of stable concentrations in the 24 h and 7 days groups, being somewhat lower in the 7 days group, throughout the Sampling. The shaded area corresponds to the standard deviation of the mean. Data from Mf3-24h, samples 11–13, are not shown in this graph (*).

ative variation across the perilymph samples. Maximum concentration levels were perceived at S4 and S5. As commented previously, due to FITC-Dextran delivery issues in the cochlea spaces in Mf1-24h, FITC-Dextran concentrations for this specimen ranged between 2 and 4 µM. *Fig. 2* summarizes findings.

3.2.3. 7-days group

Mean FITC-Dextran concentrations from S1 to S10 were 2502 µM, 2464 µM, 1891 µM, 2294 µM, 1799 µM, 1725 µM, 1857 µM, 1880 µM, 1748 µM, 1410 µM. Again, S1 FITC-Dextran concentrations are high, slightly higher than group-24 hours. The sample means show a progressive decrease through sampling. However, this decrease may be overly influenced by unusual events. In our opinion, the decline could be attributed to the device failure of Mf1-7d and to the sharp decline observed after S5 in Mf3-7d. Data is summarized in *Fig. 2*.

3.2.4. Comparative analysis between groups

As the Factorial Mixed ANOVA shows a significant interaction ($F(18;90) = 2.29$; $p = 0.006$), we continue using contrast at Sampling 1 and at Sampling 2 in order to know the differences between groups. In sampling 1 (S1), statistically significant differences were observed between the 2 hours and 24 hours groups ($Z = -3.31$; $p = 0.001$) and between the 2 hours and 7 days groups ($Z = -3.22$; $p = 0.001$), but not between the 24 hours and 7 days groups ($Z = 0.27$; $p = 0.785$). In sample 10 (S10), statistically significant differences could be seen between the 2 hours and 24 hours groups ($Z = -2.54$; $p = 0.011$), while neither between 2 hours and 7 days nor between 24 hours and 7 days, statistically significant differences were found ($Z = -0.85$; $p = 0.394$ and $Z = 1.74$ $p = 0.082$, respectively). Taking into account the average of all the samplings of each group and assessing the differences between groups, statistically significant differences have been found ($F(2;10) = 10.6$; $p = 0.003$). The comparisons between groups shows a difference between the 2 hours and 24 hours groups ($Z = -3.42$; $p = 0.001$) and between the 2 hours and 7 days groups ($Z = -2.09$; $p = 0.037$), while there was no statistical difference between the 24 hours and 7 days groups ($Z = 1.45$; $p = 0.147$) (Fig. 3).

3.3. Histological results

3.3.1. 2-hours group

The electrode array is placed through the RW and into the ST in all cases with an angle of insertion between 180-270° for Mf1-2h, Mf3-2h, Mf4-2h and Mf5-2h and >270° for Mf2-2h. Based on Eshraghi classification, Mf1-2h, Mf2-2h and Mf3-2h are assessed as grade 0 and Mf 4-2h and Mf5-2h are assessed as grade 1within the first turn of the cochlea. Minimal tissue reaction is depicted within ST spaces or intracochlear structures in the basal turn *(Fig. 4)*. Regarding the apical turn, the perilymph sampling cochleostomy site is seen directly in Mf2-2h and Mf4-2h measuring 154.6μm and 319.51μm respectively. Bone chips introduced during the opening of the cochlea for sampling can be observed in the apical turn in Mf2-2h, Mf3-2h, Mf4-2h and Mf5-2h *(Fig. 5)*.

There were no remarkable histological findings that would explain the observed variation of FITC-Dextran concentrations. Special attention was paid to the opening of the CA on the ST. There were no histological findings that indicated a compromised CA. It is noteworthy in Mf4-2h, an unusual amount of bone chip was observed on the modiolus. This specimen shows higher levels of FITC-dextran once a maximum level is reached at S7.

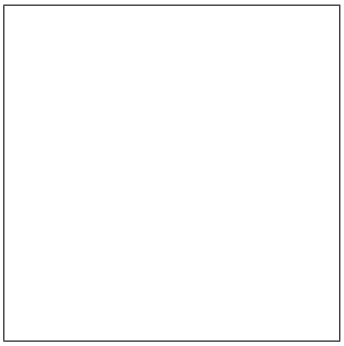

Fig. 3. Comparative sampling results from the 2 h, 24 h and 7 days group. The following graph compares the results obtained from the three groups analysed. Statistically significant differences between the 2 h and 24 h groups **(*)** ($p = 0.001$) and between the 2 h and 7 days groups **(**)** ($p = 0.037$) were observed, while between the 24 h and 7 days groups no statistical differences were found.

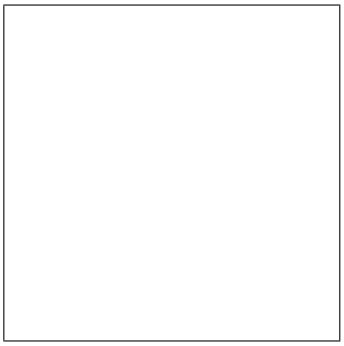

Fig. 4. Histological findings at the level of the basal turn of the cochlea in each specimen for the 2 h group.

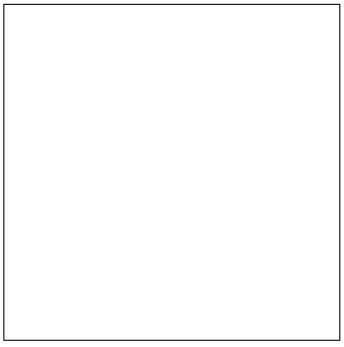

Fig. 5. Histological findings at the level of the apical cochleostomy in each specimen for the 2 h group.

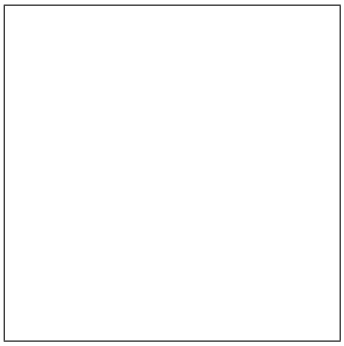

Fig. 6. Histological findings at the level of the apical cochleostomy in each specimen for the 24 h group.

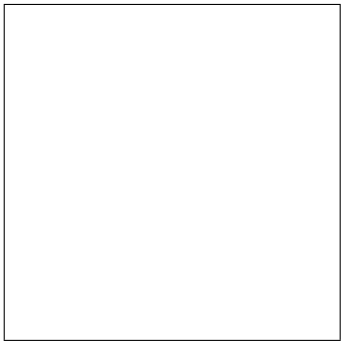

Fig. 7. Histological findings at the level of the apical cochleostomy in each specimen for the 7-days group.

3.3.2. 24-hours group

ST electrode insertion is achieved in this group with an angle of insertion ranging 180 to 270° for Mf1-24h, Mf3-24h, Mf4-24h and Mf5-24h. For Mf2-24h insertion is deeper (>270°). Insertion of the electrode showed an Eshraghi 0 for Mf2-24h, Mf3-24h and Mf4-24h and an Eshraghi 1 for Mf1-24h and Mf5-24h.

Regarding the apical area, the sampling cochleostomy is performed in the apex for all Mf except for Mf2-24h, in which it is located in the second turn; and notably in this particular case, FITC-Dextran concentrations are higher than Mf3-24h, Mf4-24h and Mf5-24h. Cochleostomy dimensions are 139.13μm for Mf3-24h, 218.29μm for Mf4-24h and 214.03μm for Mf5-24h. The location of cochleostomy is slightly different for Mf5-24h being the most apical. In this case FITC-Dextran concentrations are lower than Mf3-24h and Mf4-24h. Intracochlear bone chips were observed for Mf2-24h, Mf3-24h and Mf4-24 (*Fig. 6*). With respect to the CA, no lumen obliteration is seen, although in Mf4-24h, CA's vessel is thickened.

3.3.3. 7-days group

Electrode array insertion is confirmed in the ST with a low degree of EIT, demonstrating Eshraghi grade 0 for Mf2-7d, Mf3-7d and Mf5-7d and grade 1 for Mf1-7d and Mf4-7d. Angle of insertion is 180-270° for Mf2-7d, Mf3-7d, Mf4-7d and Mf5-7d whereas a deeper insertion is achieved in Mf1-7d (>270°). The only finding in the basal turn is seen in Mf1-7d with the presence of glue and bone dust. The CA seems patent in all cases. It is noteworthy for Mf3-7d and Mf4-7d that it the CAś blood vessel is thickened. Indirect signs of apical cochleostomy are depicted. It is placed at the level of apex for Mf1-7d and Mf5-7d. Opening in Mf3-7d is placed in the second turn whereas in Mf4-7d it shows a fracture of spiral lamina and a second opening in the basal turn (*Fig. 7*).

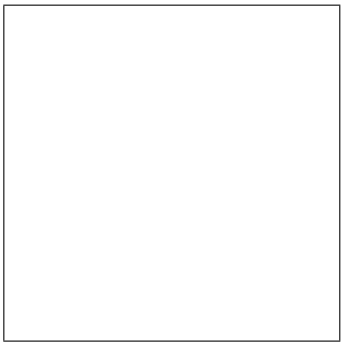

Fig. 8. Intracochlear volumes of Macaca fascicularis. The figure shows an image slice with cochlea cavities segmented by the algorithm (red for tympani, green for vestibuli and blue for media) and the attached table provides the volume sum of each cavity and fluids in the cochlea.

In Mf1-7d, disconnection of the silicone tube was observed at the level of the SS connector, which resulted in collection of perilymph without significant amounts of fluorescein in the 10 samples collected. Moreover, it is also important to highlight that the concentrations obtained in Mf3-7d were completely different. The first four samples obtained follow almost the same pattern as the other specimens. However, after S4, the flow started to be much lower. Due to the lesser fluorescein concentration witnessed while sampling, in this case, a total of 13 samples were obtained. Concentrations of fluorescein decreased from sample 5 to 12 (mean concentration: 33µM) to get again increased in S13, until 1234µM. Sample analysis was repeated to ensure there was no failure in this part of the procedure.

3.4. Intracochlear volumes of Macaca fascicularis

There is a total of 53 histological slices with 40 microns of gap between two slices (each slice has a width of 10μm). Each slice has 1232-782 pixels with a pixel size of 11.2 $\times$ 11.2 μm. Once the images are set into an image stack and converted to gray scale (8-bits per pixel), a semi-automatic procedure was applied in order to compute the volume of the different perilymphatic spaces in the cochlea (Fig. 8). Total volume for scala tympani is 4.15 mm^3, scala vestibuli is 2.81 mm^3, and scala media is 0.876 mm^3. This results in an estimated intracochlear volume of 6.96 mm^3.

4. Discussion

This experimental study shows that a model of drug delivery and pharmacokinetics using an active pump connected to an electrode array is feasible in Mf. In this work the methodology used by Salt et al. (2017) in Gp was reproduced in the Mf. Thus, the same substance, FITC-Dextran, was injected into ST of the cochlea through the RW, taking perilymph samples at the apex, under the same methodological principles and following the same technique for their concentration analysis. The surgical procedure performed in our experimental animal logically varies from that used by Salt in Gp, being adapted to the anatomy of the Mf and allowing us to reliably achieve the objectives of this study.

The cochlear anatomy of Mf has been previously described in various studies *(De Abajo et al., 2017; Manrique-Huarte et al., 2018; Manrique-Huarte et al., 2020)*. The anatomical and physiological similarity existing between this mammal and the human being, makes it possible to extrapolate some of these results from one to another *(Rask-Andersen et al., 2012; Wysocki, 2008)*. However, and despite their phylogenetic proximity, it is always important to have in mind that there could still be some particular differences between both specimens.

Our results differ depending on the group for which FITC-Dextran was delivered. In the 2 hours injection experiment, an increase in FITC-Dextran concentrations over the total of samples obtained was seen, reaching maximum concentration peaks (420-964μM) between samples 5 and 7, decreasing in successive samples, without returning to baseline. However, the results in the 24-hours and 7-days injection experiments describe a more stable behaviour throughout the 10 samples obtained, reaching a plateau with mean concentrations ranging from 1409 to 2502μM, for 7-days group and from 2144 to 2564 μM for 24-hours group, that is, lower in the 7-days injection group.

Previous studies analyzed cochlear pharmacokinetics in Gp *(Salt et al., 2017)*. This group carried out the first studies in which the distribution of a substance through the ST was measured following CI mediated delivery. Their study showed for the three groups (2 hours, 24 hours and 7 days) high dextran concentration levels in the first samples (upward curve until sample 3-4). In the 2 hour group the concentration declined along scala tympani towards the apex. This concentration gradient remained, though reduced, after 24 hours injections, and finally, almost disappeared after 7 days injections. Our study is based on the methodology described by this working group. The difference in results between the specimens here studied and Gp led us to analyze those possible determining factors affecting the results obtained. Cochlear pharmacokinetics *(Salt et al., 2009)* is mainly governed by the so-called "LADME" scheme (Liberation, Absorption, Distribution, Metabolism and Elimination of drugs). Below we will refer to each of these LADME concepts to contrast our results with those obtained by other authors.

4.1. Liberation and absorption

In this study, the release is performed through a cannula associated with a CI, so the release occurs directly into the ST of the cochlea, 4mm away from the RW, with subsequent histological verification. In this way, the absorption that would take place if the substance were released at the level of the middle ear does not condition the results obtained.

The chosen delivery method also plays an important role in cochlear pharmacokinetics. Salt et al. (2018) gathered information about the advantages offered by the release of a certain substance, directly in the perilymphatic spaces (decreasing the losses through the middle ear in the case of intratympanic administration, for example), as well as those advantages derived from a constant drug release over a long period of time, in order to favour the intrinsic pharmacokinetic mechanisms of the cochlear structure itself (mainly reliant on passive diffusion, which controls distribution of the substance to the more apical regions of the cochlea,). Indeed, this form of release by means of CI-Cannula-Pump ensures a constant delivery of drugs with respect to other techniques, for example systems using coating CI electrodes *(Manrique-Huarte et al., 2020)*. Moreover, it is also important to highlight the fact that the Eshraghi score for each of the 15 Macaca fascicularis did not exceed 1, which represents an important positive result of the surgical technique that would also have positive repercussions in the liberation process, sampling and data interpretation.

4.2. Distribution and metabolism

S1 (the first 1μL perilymph sample in the sequence of 10 samples) is a reliable indicator of the degree of diffusion that the released of FITC-Dextran has had along the ST of the cochlea until reaching the apex, since it is not influenced by the suction effect generated during sampling with capillary tubes. Statistically significant differences were found in S1 concentrations between the 2-hours and 24-hours groups ($p = 0.001$) and between the 2-hours and 7-days groups ($p = 0.001$), while no significant difference was found between 24-hours and 7-days group samples. This fact suggests that FITC-Dextran in scala tympani when delivered in the described manner, reaches a maximum apical concentration and steady state -distribution within the timeframe of 2 hours to 24 hours, i.e. the time taken to spread throughout the cochlea and to reach the cochlear apex (*Fig. 3*). The comparative statistical analysis of the mean concentrations of all samples (S1 to S10) from each group, shows statistically significant differences between the 2-hours and 24-hours groups ($p = 0.001$) and between the 2-hours and 7-days groups ($p = 0.037$), while no statistically significant differences were found between the 24-hours and 7-days groups (*Fig. 3*). This fact reinforces what has been expressed for S1, and indicates that the groups of 24-hours and 7-days tend to maintain stable concentrations during sampling.

The plot of concentration curves for the three groups shows different behaviour by group. In the 2-hours group, S1 shows a low concentration in the cochlear apex, to subsequently undergo a clear increase, followed by a slight decrease, which somewhat emulates the path observed in Gp. Salt et al hypothesize that CSF flow plays a role in the lavage caused by the flow of CSF entering through the CA. This trend is not seen in the 24-hours and 7-days groups. In these two groups, high concentrations are observed from S1, above 2000 μM,. There are several factors that could be considered when interpreting these differences in the distribution and metabolism of our working groups:

4.2.1. RW leakag

Feijen et al. (2004) mentioned the importance of the RW position in relation to the resistance of the CA to fluid flow. The inward displacement of the RW membrane is thought to increase fluid resistance. Salt, A. et al. (2017) demonstrated the importance of achieving a correct sealing of the RW to avoid retrograde flow currents, which can be hindered by the small size of the injection area. In their analysis, they established that fluid leaks at the injection site had a great influence on the fluorescein levels obtained at the apical level.

The placement of the CI through the ST could produce this change in the RW membrane position, influencing the intracochlear fluid flow and, therefore, the differences found between the two animal models. On the other hand, the opening of the RW for the insertion of the CI electrode array can enable the elimination of a substance after its administration at the level of the basal scala of the cochlea. The possible leakages that can be generated by an incorrect sealing of the RW, after the placement of the implanted device, play an important role in this aspect *(Salt and Plontke, 2018)*.

In our animal model, the sealing was carried out in 3 different ways: either using only fascia, only glue or both together fascia + glue. Out of the 15 animals in the study, leakages were observed through the RW in a total of five Mf, all of them belonging to the 2-hours group (Mf1-2h, Mf2-2h, Mf3-2h, Mf4-2h and Mf5-2h), , determined by observation of FITC-Dextran at RW niche level.

4.2.2. Perfusion, passive diffusion and reservoirs

In this experiment, the volume flow generated by the infusion pump (2μL/h) was maintained for all the groups.

In this study, 2μL per hour were injected in a pulsatile flow. It has been demonstrated that this kind of perfusion, rather than a continuous flow, reduces significantly the high basal concentrations while maintaining almost same apical concentrations. This fact is considered to be especially relevant to optimize concentrations within a therapeutic window *(Moudgalya et al., 2019)*. However, when analyzing the distribution of a substance injected intracochlearly, it was concluded that not only the volume flow generated by the drug infusion pump, but also the passive diffusion,

inherent to the cochlear structure, plays a fundamental role. This fact was corroborated, as previously mentioned, by showing that the substance diffused better towards more apical regions of the cochlea in those cases in which the release of the drug was done in constant amounts and for longer periods of time, as opposed to those cases in which large amounts of volume were injected, in a shorter space of time *(Salt et al., 2018)*. Given that the distribution of the drug is dominated by passive diffusion, it will be strongly affected by the possible losses to compartments adjacent to the scala and other structures described in Gp, but also by the characteristics of the injected substance itself *(Salt et al., 2018)*. We can also consider anatomical reasons for observed differences; particularly the different patency and dimensions of the CA, being longer and narrower in macaques than in rodents, and thus CSF potentially having a lesser influence on cochlear pharmacokinetics. Furthermore the different dimensions of the inner ear could play a role *(Burton et al., 2019), as could* the probable existence, already demonstrated in rodent models, of solute exchange between the scala tympani and other nearby structures, such as the spiral ligament *(Moudgalya et al., 2019; Salt and Hirose, 2018)* or the scala media or the modiolar space *(Rask-Andersen et al., 2006)*. This passive diffusion towards other reservoir compartments could be greater in our animal model, buffering a reduction in the concentrations obtained throughout the sampling process.

4.2.3. Anatomical and physiological differences of the CA between species

Salt et al. (2017) demonstrated that one of the major conditions of cochlear flow is the entry of CSF to the basal scala through the CA, which would generate a mixing effect that would increase distribution toward the apex. The results obtained in our study may be conditioned by the anatomical characteristics of this structure in Mf. The CA is longer and narrower in humans *(Burton et al., 2019)*, compared to rodents, and the volume of perilymph in the ST is greater, being about 24 times greater and 3 times smaller the volume of the inner ear in macaques than in rodents and humans, respectively *(Burton et al., 2019)*. Thus, in humans the influence of CSF entry on the pharmacokinetics of the cochlea may be minor. The volume of CSF that would reach the basal scala of the cochlea, as well as the mixing effect that CSF could generate, would be smaller, and therefore more time would be required to reach a peak of maximum concentration at the apical level. Subsequently, the concentration of fluorescein may be more easily washed-out in Gp. Given the anatomical similarities between humans and Mf, the reduced CA impact could be considered as a factor for Mf, although more studies should be carried out in order to discern the exact dimensions of these anatomical structures. A detailed bibliographic review was carried out in order to find the possible anatomical relationships between the Gp and the Mf. However, to our knowledge, there are not many documents detailing an inner ear anatomical comparison of these two animal specimens. However, in 1968, Neiger carried out an experimental study, in order to know in depth the anatomy of the inner ear of the Macaca rhesus, with special emphasis on the understanding of the dimension and function of the CA. The bibliographic review carried out, as well as our own results, offers a comparison between the dimensions of this structure in the three species relevant to this work: Gp, Macaca and human being. It is important to highlight that Mf and Macaca rhesus are closely related, belonging to the *Cercopithecidae* family (*Arthropoidea* suborder), with very similar morphological characteristics between them and sharing order and suborder with the human being. Thus, Neiger concluded by calculating the quotient of the length and the diameter of the CA of each of these species, that the CA of the Gp is greater (0.1) with respect to the Macaca rhesus and the human being (0.019-0.005 and 0.016-0.006, respectively). This statement may be consistent with the results obtained in this study, explaining the minor influence that the CA could have on the cochlear pharmacokinetics in Mf.

4.2.4. Location and performance of the apical cochleostomy

The opening of the apical cochleostomy could help to generate a flow from the injection site (RW-base of the cochlea) to the apex. The opening of the cochleostomy plays a fundamental role at the apical level, as it could generate a “suction effect” that conditions an apical flow of perilymph, which would otherwise be non-existent in the case of not having this cochlear opening.

In order to avoid these invasive techniques, some authors carried out studies with the aim of determining indirect measurement methods which allow the preservation of the structural integrity of the inner ear *(Moudgalya et al., 2019)*. In 2013 Haghpanahi et al. carried out a study in mice in which they released artificial perilymph with contrast agent at the basal turn of the tympanic scala in order to, by means of a non-invasive technique (micro-computed tomography), be able to monitor and quantify the different fluid-filled cochlear spaces, as well as the radial and longitudinal distribution that a certain substance presents when it is injected directly into the inner ear, without the alteration that the apical cochleostomy entails. Their results are in accordance with those obtained by other authors, showing that, regardless of the opening of the cochleostomy at the apical level, passive diffusion is the dominant mechanism in drug distribution, not only through the cochlea, but also to the different adjacent compartments *(Haghpanahi et al., 2013)*. This study was continued by Moudgalya et al. (2019) extending that technique with 3-D image registration and then enabling higher spatial resolution extractions of concentration. Although these non-invasive techniques seem to generate a lesser disturbance of the physiological mechanisms of the cochlea, the results obtained when analyzing concentrations are in accordance with those obtained after perilymph sampling, so the aforementioned “suction effect” in rodent models may not have significant repercussions in cochlear pharmacokinetics *(Moudgalya et al., 2019)*.

However, several other authors state that when the cochlea is properly sealed, the volume and intracochlear flow generated through the CA is very low, while when the otic capsule is perforated, the perilymph is quickly replaced by CSF entering into the basal turn of the ST through the CA *(Mynatt et al., 2006; Salt and Hirose, 2018)*. However, it has also been demonstrated that the perilymph volume changes produced while increasing perilymph osmolarity (with FITC-Dextran, for example), generate CSF fluid movement across the CA towards the ST to reach compensatory hydrostatic pressures *(Salt and Hirose, 2018)*.

In all of our cases, an apical cochleostomy could be performed and, with the exception of two cases (Mf1 in the 24 hours group and Mf1 in the 7 days group), both due to device failure, perilymph samples were obtained in the expected volumes (approximately 1μL for each sample obtained). However, the time frame for the obtainment of the 10 samples was variable, ranging from 13 to 37 minutes in the 2-hours group (mean: 27.4 minutes); from 24 to 117 minutes in the 24-hours group (mean: 64.8 minutes); and from 45 to 78 minutes in the 7-days group (mean: 66.6 minutes). Concerning the time elapsed while sampling in the 7-days group, it is also important to highlight the larger number of samples taken: 11 samples in Mf1-7d and 13 samples in Mf3-7d. Histologically, it was verified that the cochleostomy was performed in the expected location, except in Mf2-24h, in which it was performed on the second cochlear turn. In this animal, the concentrations of FITC-Dextran were higher than in the rest of the studied animals. We estimate that these concentrations obtained could be due to the greater proximity to the release site. The histological findings detected at the apex, such as the presence of bone chips corresponding to the needle drilling manoeuvres, did not seem to

influence the results in FITC-Dextran concentrations, although their presence and the size of the cochleostomy could have an influence on the length of sampling time.

4.2.5. Cochlear fluid spaces volumes

Estimation of intracochlear perilymphatic volume in our model is 7.15mm^3.

Within this concept of volume, it is important to highlight the space that the cannula of the CI occupies in the ST, with respect to the dimensions of this structure in Mf. This fact could affect the speed of distribution of the perilymph to the apical region. The length of the cannula in the Mf covers exclusively the basal turn, so the diameter of the ST would only be affected in this area.

4.3. Elimination

It has been shown that the rate of elimination of the substance from the perilymph is highly influential on the concentrations reached in the perilymph at the level of the cochlear apex. As Salt et al (2017) describe, if the elimination occurs more rapidly than the substance diffuses, it will not reach the cochlear apex in significant concentrations. *Moudgalya et al. (2019)* confirmed this finding demonstrating that concentrations profiles at the basal turn of the cochlea are similar to the infused concentrations and show a significant drop when approaching to the apex, and being there limited due to the clearance mechanism, even with longer infusion times. This fact could also be conditioned by the different dimensions of the Gp cochlea and of the Mf, which may entail changes in the volume of perilymph and the flow rate. Therefore, and depending on the specimen studied, different infusion time to reach the apex at certain concentrations would be required. In addition, and as described by Salt et al. (2017), the slower the distribution throughout the scala is, the greater the impact of losses to adjacent fluid compartments such as the vasculature, perilymph, middle ear and otic capsule. It has been demonstrated that solutes in perilymph spread to most of the tissues of the inner ear (spiral ligament, spiral ganglion, canaliculi in the bony walls of the ear…) until stability is reached *(Salt and Hirose, 2018)*. In this case, the FITC-Dextran is known to be well retained in the perilymph and, therefore, the losses are suspected to be inadequate to cause a lack of distribution to the apical regions of the cochlea.

Concerning these elimination routes, it is also important to consider the extracellular space as an additional volume or sink into which drugs can distribute from perilymph *(Salt et al., 2017)*. This additional volume could act as a reservoir, leading to the obtainment of stable concentrations during sampling, particularly in the case longer time points and cases of slow sampling rates. The existence of free fluid communication between the perilymphatic and modiolar space *(Fig. 4)*, as well as communications between ST (perilymph) and Scala Media (endolymph) had been previously described *(Hahgpanahi et al., 2013; Rask-Andersen et al., 2006)*. Thus, we consider the endolymphatic sinus as another important structure to analyze in drug delivery studies, as it has been demonstrated that when perilymphatic pressure is increased by injection, this structure may collapse against the bony wall, acting then as a barrier to endolymph entering the endolymphatic sac *(Salt et al., 2004)*. The possible movement of FITC-Dextran from perilymph to endolymph could produce an increase in vestibule pressure, collapsing the endolymphatic sinus and creating then a "pseudo-reservoir" where FITC-Dextran could be retained. Then, once the cochleostomy at the apex of the cochlea is done, a decrease in perilymphatic pressure will take place, decreasing the endolymphatic pressure and reopening the endolymphatic sinus. This hypothesis together with the influence of other previously mentioned possible determining factors, such as the lower influence of CSF in the specimen here studied, could explain the stable concentrations obtained in our measurements, as well as the lack of a complete "wash-out" of FITC-Dextran from perilymph while sampling. This trend was only observed in the 24-hours and 7-days groups. We understand that a prolonged release time of FITC-Dextran favoured the filling of the aforementioned reservoirs and could explain the differences between these two groups, which, although not statistically significant, did tend to be lower in the 7-days group, where more FITC-Dextran was able to disseminate to these reservoirs.

Finally, the possible traumatic insertion of the CI can also condition the presence of leaks at different levels of the ST. In our study, all specimens were in detail histologically analyzed. The insertion was atraumatic in all cases performed, so in our case, we do not expect the results to be heavily influenced by this factor.

4.4. Complications

At 7 days, the analysis of results was more difficult to carry out given the problems encountered with the pump-cannula hardware (similar to those presented by Salt et al. with ruptures of the pump, failures of the cannula). In this study, in Mf1-7d, disconnection of the silicon tube was observed at the level of the SS connector, which resulted in very low concentration of fluorescein in the 10 samples collected. This specimen is especially interesting given that the pump had been partially emptied during that period of time, without appreciating excessive accumulation of fluorescein at the level of the RW or of the mastoid region. This could indicate that, until failure of the pump-cannula connection occurred, a small amount of fluorescein was released into the cochlea. When carrying out the sampling, mean concentrations of around 3-27μM of fluorescein were observed in the 10 samples collected, which indicates that although a "washing" of substance occurs, it does not become complete, despite cessation of substance administration.

Moreover, the concentrations obtained in the third specimen of the 7 day group were completely different. Concentrations of fluorescein decreased from sample 5 to 12 (mean concentration: 33μM), and subsequently increased in S13, until 1234μM. In our opinion, there are two main hypotheses that may explain this observation. First, the presence of bony splinters in the area of the apical cochleostomy, as well as the presence of glue (cyanoacrylate) within the ST analyzed in subsequent histological studies, could condition both the sampling and the apical distribution of perilymph with FITC-Dextran. This possible existence of a bone splinter or some glue remains that plugged the cochleostomy area, could have been displaced when carrying out the sampling. Secondly, it is possible that physiological changes of the specimen may have resulted in an increase in CSF flow at that time.

4.5. Limitations of the study

The findings of this study have to be seen in light of some limitations. We have identified three major limitations that could be addressed in future research. Firstly, the methodology here used to calculate the cochlear volume of the Mf should be interpreted with caution. The volume calculation cannot be more than a rough estimate given the tissue shrinkage that occurs during histological processing rather than quantifying in live tissue. Further studies are needed so as to obtain a more accurate measurement.

Secondly, the comparison between our results and those obtained from other previous studies requires cautious interpretation due to the difference in species used. It is an assumption of the present study that that based upon apical fluid sampling, the solute distribution in the macaque would be the same as the guinea pig. Comparison with other animal models is beyond the goals of our study. It might be accounted for in future research projects.

Finally, it is always important to have in mind that despite the phylogenetic proximity and the anatomical and physiological similarity between the specimen here studied, Mf, and the human being, some significant differences between both specimens could still exist. The results obtained in this study should be interpreted in this context when it comes to being extrapolated from one to another.

5. Conclusions

Our study demonstrates a concentration gradient from base to apex of a substance delivered into the base establishes within the first two hours. This is similar to previously reported concentration profiles in the Gp. However, a longer infusion time of the substance is required to reach a maximum concentration peak at the apex of the cochlea. Moreover, the data suggest that an even concentration profile from base to apex establishes and is then maintained throughout the infusion time in the Mf. Flows during injection and during sampling may differ between Mf and Gp. In Gp we can assume flow and drug movement is dominated by the CA. This may not be true for Mf. On the other hand, it should be highlighted that the hypothesised substance exchange from scala tympani to extracellular spaces, such as the modiolar space or between the cochlear apex and the endolymphatic sinus, may play a major role in acting as a possible substance reservoir to provide an relatively flat concentration profile from base to apex during sampling. Other studies should be carried out in order to properly establish not only the exact dimensions of the CA and the other structures involved in this specimen, but also the influence that these factors may have on the analysis here mentioned.

Local administration, directly through a cannula incorporated into the cochlear implant device, supposes a more stable and uniform release of the drug, favouring the pharmacological action of the substance, as well as the reduction of possible side effects triggered by systemic administration. The extensive experience in human cochlear implant surgery, as well as the close phylogenetic proximity between Macaca fascicularis and human being, allow us to approach the future extrapolation of the results here obtained, with the possibility of treating inner ear pathologies through a combined approach of drug therapy and electrical stimulation. Probably, in the near future, the release of corticosteroids and neuroprotective or neuroregenerative substances would be two of the fields of clinical applicability in the use of cannulated cochlear implants with the use of perfusion pumps.

Declaration of Competing Interest

No conflict of interest was declared by the authors.

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Author contributions

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