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Microbubble-assisted ultrasound for inner ear drug delivery

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Keywords: sonoporation, inner ear, drug delivery, round window membrane, microbubble, ultrasound, hearing loss.

Abbreviations: ABR: Auditory brainstem response; AAV, Adeno-associated virus; CS: chitosan; CS-AuNPs: chitosan-coated gold nanoparticles; DNA: Deoxyribonucleic acid; DPOEA: distortion product otoacoustic emissions; FITC: Fluorescein isothiocyanate; HEL-OC1: The house ear institute-organ of Corti 1; HSA: human serum albumin; IGF-1: insulin-like growth factor-1; ISATA: Intensity spatial average, temporal average; ISPTA: Intensity spatial peak, temporal average; MB: Microbubble; NIHL: Noise-induced hearing loss; P407: Poloxamer 407; PRISMA: Preferred reporting items for systematic review and meta-analysis; RNA: Ribonucleic acid; RWM: Round window membrane; SNHL: Sensorineural hearing loss; US: Ultrasound; USMB: Ultrasound microbubble.

Highlights

- Strong medical need to deliver therapeutic agents to the inner ear.
- Sonoporation is an easy, promising, and safe modality.
- Optimized parameters allow for greater therapeutic efficiency.
- Sonoporation provides new possibilities to treat inner ear pathologies.

Graphical abstract

Abstract

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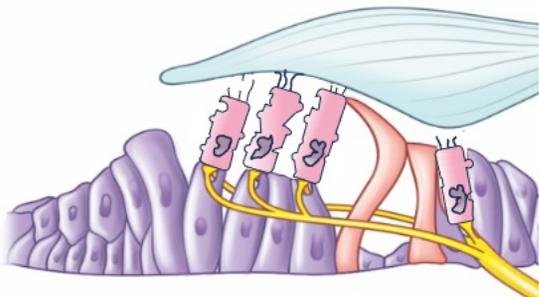
References

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Highlights

Pathologies

- HL related to age
- HL related to noise
- Menière's disease
- Congenital HL
- Auto-immune HL



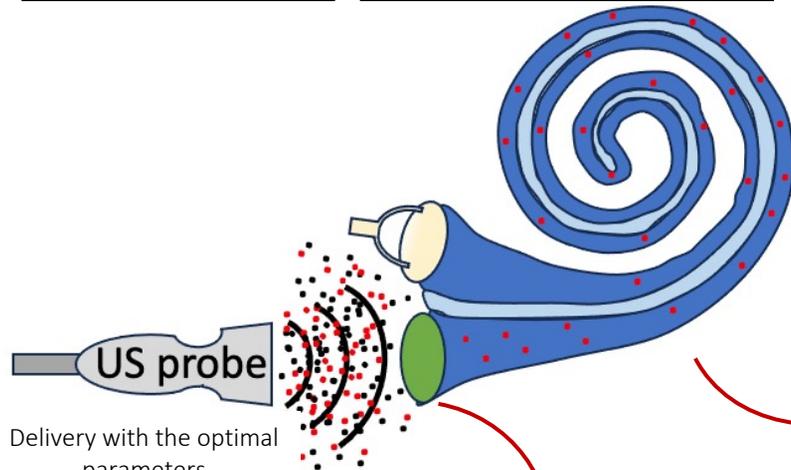
Damaged hair cells



Hearing loss

USMB

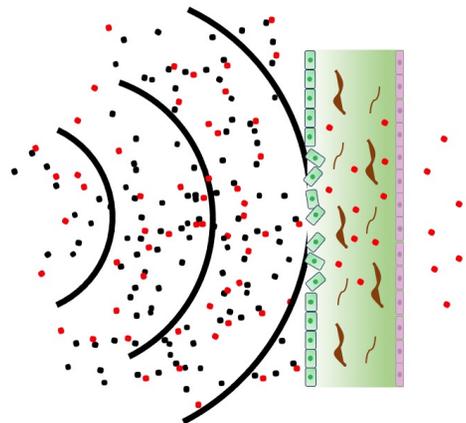
Inner ear delivery



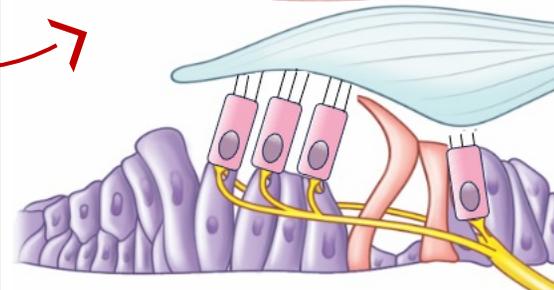
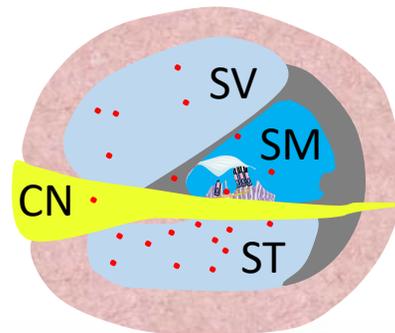
Delivery with the optimal parameters

- MBs
- Therapeutic agent

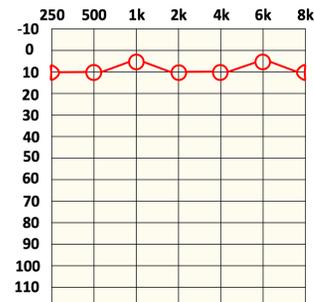
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Consequences



Repaired hair cells



Hearing restoration

Abstract

Treating pathologies of the inner ear is a major challenge. To date, a wide range of procedures exists for administering therapeutic agents to the inner ear, with varying degrees of success. The key is to deliver therapeutics in a way that is minimally invasive, effective, long-lasting, and without adverse effects on vestibular and cochlear function. Microbubble-assisted ultrasound (“sonoporation”) is a promising new modality that can be adapted to the inner ear. Combining ultrasound technology with microbubbles in the middle ear can increase the permeability of the round window, enabling therapeutic agents to be delivered safely and effectively to the inner ear in a targeted manner. As such, sonoporation is a promising new approach to treat hearing loss and vertigo. This review summarizes all studies on the delivery of therapeutic molecules to the inner ear using sonoporation.

1. Introduction

Hearing plays a crucial role in communication and perception of the environmental stimuli. The human auditory system, consisting of the outer, middle, and inner ear, as well as the auditory nerve and auditory cortex, allows for detection and perception of sound. Hearing loss, whether due to trauma, disease, or aging, is often irreversible and incurable there us a lack of targeted, effective therapeutics.

1.1. Sensorineural hearing loss

Hearing loss is the most common sensory deficit and a major public health problem [1]. The prevalence of hearing loss in the general population is estimated to be 20.3% [2]. Disabling hearing loss, which requires hearing aids, affects approximately 5% of the world's population (466 million people), 34 million of which are children (<https://www.who.int/>, accessed on 13 July 2023). Predictions for the year 2050 estimate that 10% of the population will suffer from disabling hearing loss (<https://www.who.int/>, accessed on 13 July 2023). The primary factors contributing to the increasing prevalence of hearing loss are associated with demographic shifts resulting from the growing global population and increased longevity [3]. The increasing prevalence of exposure to loud noise is a significant but avoidable risk factor for hearing loss, particularly among young people [4]. Hearing loss can affect oral language development, education, and social interaction. Hearing loss can negatively impact quality of life by reducing social interaction and can limit professional relationships due to difficulties in communication [2]. Hearing loss has been associated with cognitive decline and dementia [5].

Sensorineural hearing loss (SNHL) result from a damage located at the level of inner ear, auditory pathways, or auditory cortex. Endocochlear damage is thought to be the leading cause of SNHL and may be related to cell dysfunctions in the cochlea (sensory or non-sensory cells, neurons) or to alteration of molecular mechanisms [6, 7]. These dysfunctions may be

consecutive to congenital deafness, hearing loss related to age, noise exposure or ototoxicity. Current treatment is based on auditory rehabilitation such as hearing aids or cochlear implants. These devices provide good auditory performance for many patients. While advances in hearing aid and cochlear implant technology continue to improve the sound quality and speech understanding for patients with hearing loss [8-10], these devices do not treat the lesion site.

Much current research aims to restore auditory function by treating the lesion site via gene transfer in animal models [11]. Other approaches are aimed at improving inner ear delivery of therapeutics. Inner ear delivery of steroids is necessary for pathologies such as sudden SNHL, auto-immune inner ear diseases, and tinnitus [12-14]. Other patients require otoprotection and may benefit from inner ear delivery of therapeutics to prevent hearing loss due to cisplatin for example [15]. These therapeutics cannot be delivered directly to inner ear because of its complex anatomo-functional organization.

1.2 Inner ear anatomy and function

The inner ear is composed of the auditory (cochlea) and vestibular systems (*Figure 1*). The peripheral auditory system involves external, middle, and inner ear. Following the sound transmission by external and middle ears, the cochlea enables the mechano-electrical transduction via sensory cells [16]. An action potential will be generated at the level of cochlear nerve and transmitted to the auditory cortex via auditory pathways [16].

The cochlea is a small organ with a mean volume of 78 mm³ [17] and mean dimension of 4.4 (height) x 9.2 (length) x 7 (width) mm [18]. The cochlea is surrounded by bone in the petrous bone, one of the densest bones in the body, except at the level of round window, which is closed by the round window membrane (RWM), and at the oval window where the stapes is positioned [19]. As such, the inner ear is isolated all other organs except at the level of the RWM and stapes. The cochlea is divided into three filled compartments: (i) The middle

compartment (“scala media”) is filled with endolymph which is a potassium-rich fluid; (ii) The lower compartment (“scala tympani”) is filled with perilymph which is a sodium-rich fluid; (iii) The upper compartment (“scala vestibuli”) is also filled with perilymph. The endolymph is produced by stria vascularis, located on the lateral wall of the scala media. The endolymph is connected to the sacculle by the ductus reuniens. The volume of perilymph is around 158.5 mm³ (including the cochlea and semi-circular canals) and 34.0 mm³ for endolymph [20]. The perilymph is connected between the scala vestibuli and the scala tympani at the apex of the cochlea at the helicotrema. There is no direct communication between endolymph and perilymph, as there is a perilymph–endolymph barrier composed of a tight junction of cells that maintain the electrolyte composition of endolymph [21]. The organ of Corti is the sensorineural organ, based on the basilar membrane, and is located in the scala media with a typical length of 32 mm from base to apex [22]. The organ of Corti is comprised of sensory cells and support cells (e.g., Deiters’ cells). Sensory cells include inner hair cells (approximately 3500 per inner ear) and outer hair cells (approximately 12,000 per inner ear) [7]. High-frequency sounds are detected in the basal region of the cochlea and low-frequency ones sounds are detected in the apical region of the cochlea, near to helicotrema [23].

When the stapes vibrates the perilymph via the membrane of the oval window, the basilar membrane undulates in response. The cilia of the outer hair cells move horizontally, causing them to depolarize and then contract. This lowers the tectorial membrane, inducing depolarization of the inner hair cells and release of glutamate into the synaptic space within the auditory nerve fibers. This initially mechanical signal is then converted into an electrical signal.

The cochlea rolls up approximately 2.5 turns over a length of 34 mm around a central bony core (“modiolus”) [24]. Within the modiolus, there are approximately 35, 0000 spiral ganglion neurons, which transmit the auditory information to the subsequent auditory pathways [7].

Targeted delivery of therapeutics to the inner ear (e.g., to protect or restore inner or outer hair cell function) is needed to treat SNHL. However, the complex anatomy and function of the inner ear makes such targeted delivery of therapeutics very difficult.

1.3. Drug delivery targeted to the inner ear

The neurosensory tissues of the cochlea have a very limited capacity for repair. Inner hair cells, outer hair cells, and cochlear neurons do not regenerate, making SNHL permanent [25]. Moreover, the inner ear has the particularity of being an organ that is isolated from the rest of the human body. Although a small concentration of corticosteroids (i.e., 0.030 - 0.040 mg/mL) in perilymphatic fluid is sufficient to obtain beneficial therapeutic effects [26], it remains challenging to deliver even this small amount to the inner ear.

Therapeutic molecules can be delivered to the inner ear either systemically (intravenously or orally) or locally (*Figure 2*). Local delivery can be achieved using different routes such as transtympanic injections, or injections through the otic capsule. The otic capsule approach requires opening the temporal bone through the semi-circular canals, utricle, or cochlea [27]. The otic capsule approach is currently used only research in animal models and has not been clinically validated [28]. Moreover, the otic capsule approach carries a high risk of damage to the inner ear and can lead an irreversible SNHL [29, 30]. It is also possible to access the inner ear by opening the RWM (an approach used for cochlear implantation) [31, 32] to inject corticosteroids [33, 34] or by opening the endolymphatic sac to administer corticosteroids in Menière's disease [35].

The intravenous and the transtympanic routes are the most commonly routes used to deliver therapeutics in humans. The intravenous (systemic) approach has the disadvantage of acting on the rest of the body, thus inducing off-target effects of therapeutics on healthy tissues. For example, the use of high doses of corticosteroids is associated with risks of osteoporosis,

hyperglycemia, diabetic decompensation, weight gain, and gastritis [36]. The inner ear is supplied by a solitary labyrinthine artery unique from the anterior inferior cerebellar artery, representing the only blood vessel that reaches this region [19]. Moreover, the inner ear is protected by a blood-labyrinth barrier, located in the stria vascularis, similar to blood-brain barrier that separates the cells of the inner ear from the circulating fluids and tissues of the rest of the body [37]. The endothelial cells lining the cochlear blood vessels are tightly bound by tight junctions to prevent any infiltration of blood cells, macromolecules, or serum from the capillary into the inner ear tissues [15]. The stria vascularis plays a key role in the barrier. The stria vascularis is a highly vascularized tissue, which generates and maintains the unique ionic composition of the endolymph in the scala media. It enables the passage of small cationic molecules, such as corticosteroids or gentamicin, which can diffuse into the organ of Corti [38]. Increasing the permeability of the blood-labyrinth barrier to increase the diffusion of therapeutic molecules is not an option. This would lead to protein leakage from the endocochlear spaces in the stria vascularis into the bloodstream, with a consequent risk of deafness [39]. The precise location of this barrier remains poorly defined, and several additional barriers separate the fluid compartments of the inner ear from the capillaries of the vascular system [21, 39].

The transtympanic approach is feasible in the clinic, following local anesthesia. This approach is used in cases of sudden deafness, acoustic trauma or Menière's disease [40], mainly using corticosteroids and gentamicin. In the transtympanic approach, the therapeutic molecule is injected into the middle ear through the tympanic membrane. The injection is usually made postero-inferiorly, in front of the supposed projection of the RWM, which is not visualized. The injected solution is distributed in the tympanic cavity and diffuses passively [40] through the RWM to the perilymph of the tympanic ramp, and secondarily through the oval window membrane via the annular ligament [42], to the perilymph of the vestibular ramp. The RWM is a semi-permeable membrane playing a major role in the physiology of hearing for sound

transmission. [43]. The RWM is 70 μm thick and composed of 3 layers: outer epithelial layer, middle fibrous layer, and inner epithelial layer [44, 45]; the surface area is 2.5 mm^2 [46]. The outer epithelial layer is the main layer, which serves as a barrier from the inner ear to the middle ear. It contains low cuboidal cells with tight junctions at their surface [47]. A continuous basement membrane is located between outer layer and middle layer [44]. In contrast, the inner epithelial layer lacks continuity of the basement membrane and has loose junctions [48]. The membrane is thicker at the edge than in the center, where it attaches to the otic capsule [49]. Low molecular weight substances ($<1,000$ Da) passively diffuse across the RWN, while high molecular weight substances ($>10,000$ Da) require active transport, such pinocytosis [43, 50]. Human cadavers exhibit a pinocoy coefficient for Dexamethasone sodium phosphate (corticosteroid) of 1.44×10^{-2} cm/hr [51]. Some permeability factors have been highlighted: molecular weight, electrical charge (cationic molecules pass more easily than anionic molecules), RWM thickness (RWM will thin with age [52, 53]), and the state of the RWM (e.g., inflammation, liposolubility [43]). Moreover, once the molecules have crossed the RWM, they diffuse slowly into the scala media, induced by the minimal flow of the cochlear fluids [54, 55]. Passive diffusion along the scala tympani is limited by the structure of the cochlea, which is a relatively long, narrow tube with a cross-section that gradually decreases from the RWM at the base to the apex. This results in an uneven distribution of therapeutic molecules within the inner ear, with higher concentrations of therapeutic molecules at the basal turn than at the apex of the cochlea [56].

Previous studies have demonstrated that the transtympanic approach is more effective than the systemic approach in delivering therapeutic molecules to the inner ear [57, 58]. Indeed, a meta-analysis showed better hearing recovery with transtympanic injection of corticosteroid in cases of sudden deafness with an odd-ratio at 2.05 (95% CI=1.38-3.03, $p=0.4$) [59]. This technique has the advantage of fewer off-target effects than systemic injection, although otalgia,

dizziness, tinnitus, or tympanic membrane perforation may occur post-injection (incidence is approximately 1.6%) [60, 61]. However, the effectiveness of transtympanic delivery is unclear, and results in terms of dizziness reduction or hearing recovery are highly variable [62-64]. Indeed, the inner ear delivery through the RWM depends not on the diffusion of the therapeutic molecules, but also on their elimination [65, 66]. The middle ear drains back into the bloodstream and the auditory tube [67]. To avoid passage of these molecules through the auditory tube, transtympanic injections are performed supine, and patients are advised not to swallow. The molecules' elimination through the auditory tube contributes to the uncertain efficacy of transtympanic administrations [68]. The inner ear drains back into the bloodstream and the cerebrospinal fluid, while the middle ear drains through the oval window and RWM [69]. The elimination half-time for dexamethasone can be rapid: 46 minutes for scala tympani and 91 minutes for scala vestibuli [70]. Gentamicin has an elimination half-time in scala tympani ≤ 220 minutes [71]. Taken together, these studies confirm the need to improve the passage of these different molecules to the inner ear, and to better control their delivery to cochlear fluids.

The pharmaceutical industry has acknowledged the urgent demand for novel therapeutic solutions to restore auditory and vestibular functions. Numerous research groups are presently focusing on therapeutic strategies to safeguard auditory function during ototoxic treatments (e.g., cisplatin), as well as exploring sensory hair cell regeneration and gene therapy as potential avenues for treatment. Some agents, like collagenase [72], histamine [73], local anesthetic phenols [74], and hyaluronic acid [75], have been used on the RWM to remove its outer epithelial cells and improve drug delivery without the need for surgery. We note that the RWM has high regenerative capacity of epithelial cells. Three or 4 weeks after collagenase delivery, the RWM could not be distinguished from the control group [72]. The basement membrane plays a vital role in wound healing and the remodeling process that occurs after tissue injury

[76]. However, these methods carry the risk of causing infections in the middle ear cavity and damaging the RWM, or even the inner ear itself [77]. Drug delivery methods to improve diffusion across the RWM have been developed, using hydrogels [78], nanoparticles [79], biodegradable disks [80], microcatheter [81] or microWick systems [82]. The RWM is an essential gateway to the inner ear, and it will be crucial to develop modalities to improve its permeability and diffusion in this delicate region.

1.4. Sonoporation

Sonoporation, also known as ultrasound stimulated (US) microbubbles (MB), or USMB, is a non-invasive and targeted drug delivery modality that offers great promise to enhance the efficacy of therapeutic molecules (e.g., chemotherapeutics, antibiotics, antibodies, nucleic acids, etc.) by improving their biodistribution in the target tissues and reducing off-target effects on healthy tissues [83, 84]. In-vivo, these therapeutics are either co-injected with MBs or encapsulated into MBs [85]. The target tissue is exposed to US (Ultrasound) after in vivo injection and sufficient accumulation of these therapeutics and MBs into this tissue. The acoustically triggered volumetric oscillations of MBs induce several local acoustic processes (e.g., pulling/pushing process, microstreaming, shock waves, microjets) close to biological barriers (cell plasma membrane or endothelial barriers), which results in their transient permeabilization to the therapeutics through the formation of membrane pores and the stimulation of paracellular and transcellular pathways [85-88]. This enhanced permeabilization thus improves the penetration and the retention of therapeutics in the target tissues. USMB is an easy-to-use and cost-effective drug delivery modality that is currently undergoing pre-clinical and clinical investigation in oncology [89], neurology [90], nephrology [91], and cardiovascular medicine [92].

For inner ear application, therapeutic molecules intended for the inner ear presently are co-administered with MBs in the middle ear [93-104]. Accordingly, the inner ear can be exposed to US using a transcanal (via the outer ear canal), transmastoid (via the mastoid, after performing a mastoidectomy), or transcranial (via the skull) approach. USMB enhances the permeability of the RWM through the stimulation of paracellular and transcellular pathways, thus facilitating the delivery of therapeutic molecules into the inner ear. The therapeutic molecules present in the middle ear can thus more easily passively cross the RWM and diffuse in the perilymph of the tympanic ramp until reaching the apex of the cochlea and mixing with the flow of fluids. Increasing the concentration of active substances in the perilymph makes it easier for them to exert their local effects.

The objective of this review is to provide a survey on preclinical studies investigating the therapeutic efficacy and safety of delivery of therapeutic molecules for the inner ear using USMB. The advantages of this proposed localized delivery to the inner ear and its difficulties will be outlined beforehand. The limitations of these investigations and future perspectives are also discussed.

2. Results

Articles have been identified using the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) recommended methods [105] using the search terms “Inner ear” and “Microbubble”, “Inner ear” and “Sonoporation”, “Hearing loss” and “Microbubble” and “Hearing loss” and “Sonoporation”. This systematic literature review was conducted by searching medical literature analysis and retrieval system online (MEDLINE; PubMed interface), Cochrane Central Register of Controlled Trials (CENTRAL; Wiley interface), and Web of Science electronic. The search began in June 2023 and was completed in August 2023.

Sixteen articles were analyzed. After review, four articles were excluded because three did not concern sonoporation procedure and one was about nephrology. A total of 12 articles were included in this review (*Figure 3*). Studies were performed in vitro or on animals. No study was performed in humans. Animal studies were conducted with guinea pigs, mice, and sheep. All animal studies were randomized control trials (*Table 1*).

2.1 What animal should be used for inner ear USMB drug delivery studies?

The animal models used in studies concerning USMB of the RWM have mainly involved murine species: guinea pig [94,95,97-101, 103, 104] and mice [96]. These animals have the advantage of being readily available and inexpensive. However, the dimensions of their inner ear are smaller than in humans, and their hearing range is at higher frequencies [106]. Guinea pigs have easy access to the inner ear, whereas in sheep, the mastoid is poorly pneumatized, making access to the middle ear more difficult [107]. Although large animals can be disadvantageous in terms of housing, management, or cost, they are more similar to humans in terms of anatomical dimensions and auditory physiology [107]. The sheep cochlea has 2.5 turns, as in humans. The structure of the RWM in sheep exhibits several resemblances to that of humans [108]. Similarly, the auditory spectrum is also more comparable to that of human,

with frequencies ranging from 100 to 30,000 Hz, while humans perceive frequencies from 20 to 20,000 Hz [109]. If one also wishes to perform analysis of endolymphatic fluids, the size of the endolymphatic spaces is larger in sheep and therefore more easily accessible to a fluid sampling [107]. For these reasons, the sheep may be considered a more appropriate animal model to design and to validate new therapeutic options for the treatment of hearing disorders.

2.2. Sonoporation protocols

Sonoporation of the inner ear is a relatively new procedure, first described in 2012 [104], for which some therapeutic molecules have been tested in animal models. The efficiency of USMB to deliver molecules to the inner ear depends on several factors, such as therapeutics and MBs concentrations, physiological properties of RWM, US parameters, probes/devices, and therapeutic scheme [110]. In vitro investigations have been done to determine the most appropriate sonoporation protocol before moving on to in vivo trials [94-96, 98].

To date, there are no established protocols in this field and no current human clinical applications, which explains the variability of protocols and animal models studied, depending on the objectives.

2.2.1. Which therapeutic molecules can be delivered?

The therapeutic molecules used depend on inner ear pathologies. Etiologies of SNHL can be noise-induced hearing loss (NIHL), sudden hearing loss, drug ototoxicity (e.g., cisplatin, aminoglycoside), radiation ototoxicity, Menière's disease, infection, auto-immune inner ear diseases, or genetic diseases. Different therapeutic approaches have been designed to treat these pathologies such as drugs (e.g., steroids, aminoglycoside), neurotrophic therapy, gene therapy, ribonucleic acid (RNA), interference therapy, cell-based therapy with embryonic stem cells [16, 33, 111-114]. Some studies have investigated inner ear delivery using sonoporation using

different therapeutic agents (see *Tables 3 and 4*). Some therapeutic molecules have a pharmaceutical role in the inner ear (e.g., dexamethasone, gentamicin, or insulin growth factor 1 (IGF-1)), while others are vectors (e.g., Adeno-associated virus, or AAV) that allow for genetic modifications [95, 98, 101, 103].

Steroids are commonly used to treat inner ear diseases such as sudden hearing loss, endolymphatic hydrops, auto-immune inner-ear diseases, acoustic trauma, and NIHL, as well as during cochlear implant surgery [101, 115]. It is an anti-inflammatory molecule, which plays a role in metabolic pathways of oxidative stress, ischemia, and inflammation. One study in normal hearing guinea pigs compared inner ear delivery of dexamethasone solution with and without USMB and found higher dexamethasone concentration in perilymphatic fluid in the USMB group (at 0.5 MHz, 3 MHz, and 5 MHz) compared to the control conditions [101]. Another study with an in vitro model and a NIHL model in guinea pigs found similar findings using hydrogel (12.5% P407) as drug carriers to obtain sustained release of dexamethasone at 1 and 7 days after USMB [95]. Dexamethasone was visualized using immunofluorescence in all turns of the cochlea, with more intensity for USMB group, especially in the basal turn, compared to the control group [95]. Audiometric testing using auditory brainstem response (ABR) demonstrated a better auditory recovery after USMB delivery of dexamethasone, as well as less cochlear damage, especially for hair cells [95].

Gentamicin is an aminoglycoside antibiotic used to treat anti-Gram-negative infections. It is known to induce ototoxic side effects [103]. Aminoglycosides rapidly increase intracellular levels of calcium and reactive oxygen species, inducing a variety of cell death processes [116]. Transtympanic injections are used to treat vestibular symptoms in Menière's disease due to the vestibular ototoxicity [117]. Menière's disease is an idiopathic inner ear disorder characterized by endolymphatic hydrops. Previous studies investigated gentamicin delivery into the inner ear by USMB, showing more diffusion in the hair cells immediately after and 2 days after USMB

exposure, compared to the control groups [98, 103]. Two weeks after the procedure, the gentamicin was delivered into the cochlea apex with USMB, and hair cells loss was observed at the vestibular (utricle and saccule) and cochlear (basal turns) levels, with more damage compared to the control condition, showing that USMB was more effective at delivering the drug [98].

Insulin-like growth factor 1 (IGF-1) is a polypeptide involved in auditory mechanisms and is a potential drug candidate for treatment of inner ear disorders [97, 118-120]. Indeed, some animal studies demonstrated the role of IGF-1 in protecting cochlear hair cells from apoptosis, to stimulate proliferation of supporting cells, and to promote regeneration of degenerated synapses between inner hair cells and spiral ganglion neurons [121-123]. The acoustically mediated delivery of IGF-1 after NIHL resulted in a 195% increase in IGF-1 concentration 2 hours after USMB, compared to control conditions [97]. Twenty-eight days after noise exposure, the USMB group had significantly greater restoration of synaptic ribbons than did the control groups in the basal turn of the cochlea, but not in the second turn. These results suggest that USMB enhanced the therapeutic efficacy of IGF-1 for the treatment of NIHL, and that this combination resulted in a superior therapeutic effect against noise-induced cochlear damage, including outer hair cell loss and reduction in the number of synaptic ribbons [97].

Half of hearing loss in children is attributable to genetic causes [124]. Gene therapy that targets mutations represents a promising therapeutic strategy to treat genetic hearing loss. The cochlea is an ideal organ for local gene therapy due to its isolation from surrounding tissues [125]. Gene therapy presents an avenue to reinstate and/or protect the functionalities of inner ear cells. A variety of strategies can be designed for inner ear gene therapy, aimed at treating SNHL. These strategies include rectifying genetic mutations, conserving and thwarting the deterioration of distinct cell types (e.g., hair cells, spiral ganglion neurons, supporting cells)

[126]. Viral [127] or non-viral [128] vectors are used to deliver functional deoxyribonucleic acid (DNA) to repair defective genes in dysfunctional cells in the inner ear [129]. Replication-defective AAV is the main viral vector used for such applications [130, 131], and contains the sequence of the desired therapeutic gene [132]. While several AAV serotypes have shown effective transduction to inner hair cells, the infection rates of outer hair cells remain low [112]. Moreover, the transduction efficiency of conventional AAVs for cochlear supporting cells is also limited [133]. To enable complete restoration of hearing with inner ear gene therapy, a viral vector with higher transduction efficiency is needed [134, 135]. Main administration techniques are direct injection through the RWM or cochleostomy with a gelatin sponge in the RWM to reduce trauma [11]. The delivery of AAV2/Anc80L65 with harmonin plasmid resulted in promising results in Usher syndrome animal models [136-138]. Compared to viral vectors, MBs possess larger loading capacity of therapeutic nucleic acids, including antisense oligonucleotides, DNA fragments, and even entire chromosomes [139]. Thus, USMB could be a promising modality to increase gene delivery into the inner ear. The use of MBs with plasmid DNA was assessed in vitro on HEI-OC1 (The house ear institute-organ of Corti 1) cells [102]. The transfection efficiency was significantly higher in USMB group compared to control group (plasmid DNA without USMB) [102]. In another study, USMB was compared to direct delivery through cochleostomy [100]; while the cochleostomy group yielded a higher level of green fluorescent protein expression, cochleostomy is a riskier method of access to the inner ear, with irreversible auditory and vestibular damage.

To ensure the inner delivery of such therapeutic molecules, US should be applied close to the RWM; as such, a transcanal, transcranial or transmastoid approach is needed. This efficacy of these approaches depending on the animal models. For guinea pigs, the tympanic bulla needs to be opened to visualize the RWM [94, 95, 97-101, 103, 104]. For sheep, a mastoidectomy is required to expose the RWM [93]. Depending on the anatomy of the animal

models, the tympanic bulla, mastoidectomy cavity, or the middle ear were filled with the solution containing the MBs and/or the therapeutic molecules.

2.2.2. Which microbubbles are used?

Tables 2 and 3 show the different MBs used in the in vitro and in vivo studies. MBs consist of a gas core surrounded by a biocompatible shell (i.e., lipid, protein, or polymer shell) [140], to reduce surface tension and gas diffusion, which stabilizes the gas core. MBs typically have a diameter ranging from 1 to 10 μm . In addition, MBs can be covered with targeting ligands (e.g., antibodies) which bind specifically to target cells. MBs are widely used in US imaging as contrast agents for diagnosis of pathologies [141]. Contrast-enhanced US imaging is an effective, mobile, and inexpensive method that provides high-resolution images of tissues in real time. In therapeutic use, MBs can enhance the efficacy of thermal ablation of target tissue using high intensity focused US therapy.

The MB oscillation properties used in sonoporation are described below [2.2.3.]. When close to biological barriers (e.g., cell plasma membrane or endothelial barrier), the MB oscillations significantly increase their permeability to therapeutic molecules through the formation of membrane pores and/or the stimulation of paracellular and transcellular pathways. MBs can be co-injected with drugs or loaded with drugs for sonoporation of the inner ear. This co-administration approach is likely to be the fastest way to implement sonoporation in the clinic because it uses drugs and MBs that are already clinically approved. Previous studies comparing biotin-FITC (Fluorescein isothiocyanate) delivery to the inner ear with MBs but without US to delivery without MBs or US [103, 104] revealed no significant difference in fluorescence level detection [104]. In a later study [96], simple soaking of the RWM with MBs in the absence of US exposure did not change the RWM composition. This suggests that the presence of MBs alone does not enhance RWM permeability. Drug-loaded MBs may have

greater therapeutic potential, with a local delivery with US exposure. However, this approach requires a deep investigation of drug-loaded MBs in order to obtain validation from health authorities for clinical use. The physico-chemical and pharmacological properties of MBs play a major role in the efficacy of USMB to deliver therapeutic molecules to the target tissue [142].

Homemade albumin-shelled MBs have been used in numerous *in vitro* and *in vivo* studies [94, 95, 98, 101-104]. This homemade production makes it possible to control the physico-chemical properties of MBs and therefore their response to US [143], to enhance the efficacy of acoustically mediated drug/gene delivery. Albumin-shelled MBs are created with concentrations of human serum albumin (HSA) that can be adjusted to produce different MB sizes. These MBs are generated by sonication of a mixture of albumin and perfluorocarbonyl gas (e.g., perfluorobutane, perfluoropropane). Albumin contains negative charges, so the surface potential of its shell is less than zero and can attract positively charged molecules. MBs < 2 μm are more susceptible to destruction under constant US power density [102], so the use of larger MBs is preferred. Larger MBs are resistant to US destruction and helped to enhance the transfection efficiency of auditory hair cells at a constant US power density [102]. *In vitro* study on the effect of different IGF-1-coated MB sizes (ranging from 1.3 to 3.0 μm) showed that larger MB sizes delivered the IGF-1 more effectively than did smaller sizes. An *in vivo* study with guinea pigs showed that IGF-1 delivery into the inner ear was 15.9 times greater in animals treated with USMB than in control animals treated without USMB [97]. Using biotin-FITC as model drug and guinea pig animal models, a preclinical study showed that USMB with albumin-shelled MBs can improve the inner ear biotin-FITC delivery via the RWM by 3.5 to 38 times, compared to the simply soaking the RWM with biotin-FITC [94, 103]. In another study [102], various-sized albumin-dextran MBs were generated by adjusting the concentration of albumin or dextran in MB composition. A 330% increase in the size of albumin-shelled MBs (from 0.66 to 2.83 μm) enhanced plasmid DNA transfection to HEI-OC1

cells by 54.2%; a 250% increase in the size of albumin-dextrose-shelled MBs (from 1.39 to 3.47 μm) enhanced plasmid DNA transfection to HEI-OC1 cells by 66.8%.

SonoVue[®] are phospholipid-shelled MBs containing sulfur hexafluoride (Bracco Research SpA, Italy) [144]. The shell consists of a thin and flexible monolayer of amphiphilic phospholipids with the hydrophilic surface on the outside and the hydrophobic surface on the inside. This biocompatible shell stabilizes the gas within the MBs, and its flexibility enables the MBs to change size and shape easily. Their diameter distribution ranges from 0.7 to 10 μm , with a mean diameter of 5.7 μm . These MBs have been used in preclinical studies for acoustically mediated drug delivery in the inner ear at concentrations of $1\text{-}5 \times 10^8$ MBs/mL [96, 97, 99, 101]. In one study [101], SonoVue[®] and albumin-shelled MBs were compared; results showed that dexamethasone delivery increased by 300-1120% with SonoVue[®] MBs and 240-790% with albumin-shelled MBs, compared to the control conditions. The trans-RWM delivery of biotin-FITC (used as permeability tracers) in guinea pig model revealed that delivery efficiency was > 700% higher for the USMB groups immediately after 3 or 5 exposures than for the control group (MBs without US exposure) [99]. Another study found a 240-1120% increase in dexamethasone delivery efficiency after USMB, compared to the control group (MBs without US exposure) [101].

Vevo MicroMarker[®] are phospholipid-shelled MBs, comparable to SonoVue[®] MBs in composition (Bracco Research SpA, Italy). The MB is filled by a mixture of nitrogen and perfluorobutane surrounded by a biocompatible shell, which is composed of polyethylene glycol, phospholipids, and fatty acids. The median diameter ranges from 2.3 to 2.9 μm . They have been used at a concentration of 2×10^8 MBs/mL in a preliminary study in sheep to establish USMB innocuity for the inner ear [93]. More studies are needed to establish the effectiveness of these MBs to deliver therapeutic molecules in the inner ear. However, in previous in vitro

studies, Vevo MicroMarker[®] MBs appeared to be more efficient for drug and gene delivery than were SonoVue[®] MBs [145].

Definity[®] MBs are injectable phospholipid-encapsulated perfluoropropane MBs (Lantheus Medical Imaging, USA). Once fully activated by shaking (VIALMIX[®]), the vial contains a maximum of 1.2×10^{10} perflutren lipid MBs/mL, which is considerably higher than for SonoVue[®]. Up to 98% of Definity[®] MBs have a diameter of $< 10 \mu\text{m}$ and a mean size of 1.1–3.3 μm . The acoustically mediated delivery of AAV encoding green fluorescent protein using Definity[®] MBs in guinea pigs showed that the transduction efficiency of cochlear hair cells was significantly improved compared to the control group [100].

2.2.3. Which US devices and acoustic parameters used?

The effectiveness of acoustically mediated drug delivery depends on US parameters, including center frequency, duty cycle, acoustic intensities, and exposure time. Several investigations showed extensive optimization of the US parameters for efficient and safe in vitro and in vivo drug delivery. The results are summarized in *Tables 2* and *3*. The choice of center frequency depends on the MB size, its resonance frequency, and the depth of RWM to be reached [102]. The center frequency used for USMB of inner ear ranges between 666 kHz and 1 MHz in in vitro studies [94-96, 98, 102] and between 666 kHz and 5 MHz in in vivo studies [90-101, 103, 104]. In most of studies, a center frequency of 1 MHz has been used for acoustically mediated drug delivery [146]. The duty cycle (i.e., the percentage of time that an US device is transmitting acoustic waves [147]), ranges from 40 and 50 % in in vitro and preclinical studies. US exposure time varied from 1 to 5 min to prevent cell and tissue damage [93-104].

Under the influence of US, MBs can transform into cavitation-enhancing mediators, concentrating the energy of pressure waves to increase drug action and enhance the

permeability of cell membranes [148]. The acoustic cavitation of MBs can be classified into stable and inertial cavitation [149, 150]. Stable cavitation results in circulating fluid flow around the MBs, while inertial cavitation can produce shock waves [151, 152]. When inertial cavitation occurs near a solid surface [153], it results in an asymmetrical collapse, creating transient but nonlethal micropores in the cell membrane that promote drug transit [154]. Modifying US parameters changes the ratio between stable and inertial cavitation. Indeed, some in vivo study demonstrated more efficient MBs destruction given the intensity [98].

The acoustic intensity used for US has varied between 0.2 to 4 W/cm² for in vitro studies [94-96, 98, 102], and between 0.426 and 3 W/cm² for in vivo studies [93-101, 103, 104]. The greater the intensity, the greater the MB destruction [98]. Increasing intensity generally increases the cavitation effect. At 3W/cm², this may produce extreme shear forces that can break up the cells and negatively affect cell viability [96]. Compared to control, cell viability was reduced to 67.49 at 3W/cm² [96]. On the contrary, at 0.84 W/cm², there was no effect on cell death at 48h (HEI-OC1 cells) [102].

Most previous studies used commercial US probes (6 and 20 mm in diameter), while others used a custom probe (1.5 mm in diameter) adapted to the guinea pig anatomy of the external and middle ear [100]. The position of US probe at the RWM level is crucial and needs to be as close to the RWM as possible. Focusing US energy on a target point also minimizes the risk of collateral damage to non-target tissue such as the facial nerve. Another advantage of using an adapted probe is the reduction in the amount of MB solution. Because the probe is placed directly against the RWM, a small amount of solution is sufficient to fill the space between the US probe head and the RWM to ensure air-free contact, unlike the use of a larger US probe which would require filling the entire middle ear cavity [100].

Nearly all studies have used unfocused US probes for drug delivery to the inner ear, except for Zhang et al. [100], where a lab-made US probe with a focus of 2 mm was used.

Acoustically mediated drug delivery to the inner ear requires a precise positioning of the US probe in relation to the RWM. Focused US probes improve the sensitivity and axial resolution by focusing US energy to a small targeted area, such as the inner ear. In addition, their use has been shown to be more effective than unfocused transducers for drug delivery using USMB [155]. To achieve this goal, Zhang et al. [100] designed a dedicated US probe whose surrounding area comes in contact with the RWM, enabling a lens to be placed 0.5-1 mm from the RWM. At present, there are no comparative studies regarding focused vs. unfocused probes for USMB drug delivery to the inner ear.

2.3 What are the ultrastructural consequences of USMB on the inner ear?

Investigation of ultrastructural consequences of USMB on the inner ear using optical microscopy is important to understand the molecular and cellular mechanisms of acoustically mediated drug delivery, as well as putative tissue damage and other off-target effects on healthy tissues [156]. As discussed above, the transit of therapeutic molecules through the RWM may entail multiple cellular processes, but the outer epithelial layer is considered to be the primary barrier that regulates the passage of therapeutic agents and directly influences membrane permeability [43, 50].

In one study, the RWM was examined immediately after USMB and showed reversible visible damage in the treated region, while other regions appeared normal [100]. USMB treatment induced distinct degrees of heterogeneous pore-like openings on the epithelial surface, ranging in size from 100 nm to several microns, with some regions exhibiting a disruption of tight junctions between epithelial cells [99]. MB cavitation led to different degrees of disruption in the outer epithelial cells, causing the formation of pits of varying sizes and a notable loss of microvilli [99]. The increase in RWM permeability is ascribed to alterations in tight junctions and an enhanced permeability of the plasma membrane of outer epithelial cells,

leading to an overall increase in transepithelial permeability [104]. If the continuity of the outer epithelial layer was disrupted, such changes did not extend to the deeper layers [99, 100]. The treatment maintained the integrity of the basement membrane, safeguarding the RWM from USMB damage and ensuring its subsequent regeneration [76, 99]. One month after USMB, microscopic study showed complete healing of the outer layer, with regrowth of the microvilli [99]. Therefore, the reversible damage is specifically limited to the outer epithelial layer of the RWM without affecting deeper layers [100].

The histological changes observed on the RWM are similar to those described on the blood-brain barrier. Indeed, the exposure of blood-brain barrier to USMB resulted in transient molecular and tissue changes including the disruption of tight junctions, the stimulation of transcytosis, and the release of inflammatory molecules [157; 158]. However, the comparison of histological changes between the blood-brain barrier and the RWM stops there because the drug delivery protocols, including US parameters (*e.g.*, center frequency, duty cycle, pulse repetition frequency, acoustic pressure exposure time, *etc.*), MB related parameters (*e.g.*, type, dose, administration route, *etc.*), and therapeutic scheme, are different.

2.4. Therapeutic safety

2.4.1 What about hearing conservation?

Preclinical studies have also investigated the safety of USMB, generally finding no ototoxic effect on cochlear function. All in vivo studies included objective audiometric tests with ABR and Distortion Product OtoAcoustic Emissions (DPOAEs). The acoustically mediated delivery of biotin-FITC did not induce ototoxicity immediately after or 2 months after treatment [93, 99]. Four weeks after USMB treatment, cochlear sensory epithelial surface from guinea pigs exhibited no notable hair cell damage under confocal microscopy after immunofluorescence staining [95, 99]. In addition, ABR and DPOAE thresholds (from 4 kHz

to 64 kHz) did not differ before and 4 weeks after acoustically mediated delivery of dexamethasone and 12.5%-P407 [95]. The preservation of hearing thresholds was also observed when gentamicin, IGF-1, or plasmid DNA were delivered using USMB [94, 97, 98, 103].

2.4.2 What about metabolome conservation?

One study investigated metabolomic changes following the exposure of the inner ear to USMB [93]. No significant modification in metabolomic profile was observed in the perilymphatic space, indicating that the USMB procedure is safe.

2.4.3 What about vestibular function and facial function conservation?

Presently, no study has investigated vestibular or facial function following USMB procedure. With US, therapeutic molecules diffuse into the entire perilymph, whether in the cochlea or in the vestibule. It is clinically relevant to investigate instances of vertigo after USMB treatment. The second portion of the facial nerve emerges in the middle ear and is closely linked to the medial wall [159]. Because the US probe head is positioned as close as possible to the RWM during the USMB procedure, one cannot rule out that USMB does not affect the facial nerve.

In addition, no investigation has yet explored intraoperative electrophysiological responses during USMB procedure. Such investigations are appropriate to ensure the safety of USMB on facial nerve function. However, in many studies, animals were not awakened after the USMB procedure [94-101, 103, 104], making it difficult to ascertain any facial nerve damage such palsy.

2.4.4 What about warming up the inner ear?

The interaction of US waves with tissues and specially bones can generate undesirable temperature increment into the targeted region. Such temperature increment may affect the functions of the inner ear. Indeed, patients with hyperthermia (i.e., average temperature of 38.4°C) exhibit a marked reduction in transient evoked otoacoustic emissions, indicating that outer hair cells react to changes in body temperature [160]. Membrane and intracellular proteins undergo denaturation when the temperature rises above approximately 45°C, leading to the loss of their tertiary structure [161]. Guided by animal models, it is important to establish a permissible exposure temperature range for the middle ear and cochlea, relative to body temperature [162]. Moreover, the facial nerve could be damaged with temperatures > 46°C [163].

USMB induced temperature increase depends on several factors: US intensity, insonation time, duty cycle, and the number of sonoporation treatments in a session. For example, USMB using the transcranial approach induced a temperature increase from 1.25°C to 1.80°C after US exposure from 1 to 4 W/cm², respectively [98]. In a sheep model, USMB was applied at 300 kPa for 1 min and repeated three times, with a 1 min time interval between applications rather than 3 min continuously; with the interval treatment, no significant temperature increment measured at promontory was detected after USMB treatment [93]. Using the transcranial approach, USMB (4 W/cm² for 3 minutes) resulted in a temperature increase at the skull bone of 8.80°C [98]. There is also a temperature increase along the cochlea, with a higher temperature at the basal turn (2°C) than at the apex (1.2°C) after 3 USMB treatments [99]. After more than 3 USMB treatments, there was a tendency for the temperature in the tympanic cavity to be approximately 0.2°C lower than that of the cochlea [99]. The duty cycle, which is associated with the acoustic intensity, is also a key US parameter that must be considered to prevent tissue heating. All *in-vivo* studies for inner ear drug delivery exploited a US sequence including a high duty cycle (i.e., 40 or 50%) with low acoustic intensities, thus

explaining the absence of heating of the tissues of the inner ear. Future studies investigating the use of low duty cycle (i.e., < 10%) in combination with low and high acoustic intensities should be relevant for inner ear drug delivery.

3. In the future

Future investigations are needed to confirm that therapeutic agents can be delivered to the inner ear safely and efficiently using US modality. Presently, there is no standardized protocol for use of USMB in inner ear. In fact, US parameters (center frequency, duty cycle, acoustic pressure, time exposure, etc.), MB-related parameters (type, concentration, bare versus drug-loaded MB, etc.), US device and probe, and therapeutic schemes all must be optimized as function of physico-chemical and pharmacological properties of therapeutic molecules.

3.1 How to improve USMB procedure?

To enhance diffusion of therapeutic molecules through the RWM with USMB, some studies recommend the use of hydrogels [95, 97]. Hydrogels offer the advantages of being more viscous, thus improving time spent in contact to the RWM and preventing a rapid elimination through the auditory tube [165]. However, the hydrogel viscosity must be carefully evaluated and chosen, considering a balance between enhancing the resilience within the RWM and the challenge of injecting through a needle smaller than G22 [166]. To overcome such limitations, a synthetic thermosensitive hydrogel using poloxamer 407 (P407) was developed to modulate its viscosity in response to temperature changes [167] and to optimize drug formulations. Its fluid state at room temperature facilitates easy administration, while its gel state at body temperature promotes the sustained release of therapeutic molecules [168]. This unique characteristic, including local stability and prolonged drug residence, makes it suitable for diverse applications in the field of drug delivery in inner ear [169, 170]. Triamcinolone-acetonide-loaded P407 hydrogel has been shown to be an effective vector for sustained high-dose inner ear glucocorticoid delivery [171]. Administering dexamethasone loaded into P407 via transtympanic application one day before cochlear implantation led to a significant reduction in hearing threshold shifts in guinea pigs measured on postoperative day 28 [172].

The production of P407 hydrogel containing MBs was designed for inner ear drug delivery. The first studies revealed that MBs were not destroyed in this hydrogel [95]. P407 gel biodegrades slowly, and one study showed it was not completely discharged until 49 days after USMB treatment [173]. Such strategy did not cause any inflammation in the mucosa of the middle ear [95]. However, a temporary hearing loss (10 days in duration) has observed because of the limitation of ossicular chain [171].

Another option relies on exploitation of drug-loaded nanoparticles to increase their bioavailability and their long-time retention at therapeutic concentration in the inner ear [174]. Several types of biodegradable nanoparticles have been studied *in vivo* and *in vitro*: polymeric nanoparticles, lipid nanocapsules, metallic core nanoparticles, and liposomes [166]. Their nanoparticles are usually < 200 nm [175] and they are not ototoxic [176, 177]. These of magnetic nanoparticles for delivering steroids has been shown to alleviate hearing loss induced by cisplatin [178]. Nonetheless, when nanoparticle suspensions are introduced into the middle ear, they tend to be swiftly eliminated via the auditory tube because of their low viscosity. To address this for transtympanic delivery of nanoparticles, the duration of their presence in the middle ear can be extended by elevating their viscosity through methods like integrating nanoparticles into hydrogels. In addition, stable inorganic nanoparticle such as chitosan-coated gold nanoparticles (CS-AuNPs) can be used to improve the delivery of therapeutic molecules through the RWM. Among different types of metals, gold was chosen because of its stability [179]. The advantage of inorganic molecules such as titanium dioxide or gold nanoparticles is that they produce less reactive oxygen species and have a more manageable structure with improved chemical stability [180]. The association of gold nanoparticles with chitosan (CS), a natural biodegradable polymer, form positively charged nanoparticles and improve their stability [181]. These nanoparticles facilitate the loading of drug on their surface through electrostatic interactions, thus avoiding drug degradation during endocytosis and enhancing the

concentration of drug in perilymphatic fluid [96]. Nowadays, ototoxic effects of gold nanoparticles are still debated and need further study [96, 182]. A comparative analysis of the efficiency of CS-AuNPs passage through the three-layer membrane of the RWM demonstrated that the USMB-treated group exhibited higher penetration of CS-AuNPs, compared to the control group [96]. Ultramicroscopic examination demonstrated reduction of membrane proteins that play a major role in tight junctions (e.g., including zonula occludens-1 and occludin) after USMB procedure [183]; three days later, the tight junctions were restored [96].

More recently, modification to RWM permeability given molecular shape has been investigated. Diamond-shaped dexamethasone microcrystals were combined with silk fibroin to improve mucosal adhesive properties and the drug distribution in the RWM, resulting in prolonged release kinetics in the perilymph [184].

Moreover, US probe dedicated to the human ear anatomy is required for inner ear drug delivery. Such developments imply a miniaturization of the probe but also raise the possibility of visualizing the RMW using US imaging in order to guide the USMB procedure. Some authors recommended the use of a dual frequency probe (666 kHz + 1 MHz), which induced a highest perilymphatic IGF-1 concentration (60%) compared to a single frequency probe (1 MHz) [94].

The clinical application of USMB may be a worthwhile alternative to surgery. In this context, transcanal and transcranial USMB approaches were investigated in guinea pigs and resulted in a better delivery of therapeutic molecules or drug model in the inner ear compared to control conditions [98]. However, transcranial approach is more complicated in humans because pneumatization of the bone results in greater bone thickness (approximately 6 mm) than in guinea pigs (1 mm), possibly involving safety issues related to tissue heating [98, 185,186].

To increase the drug bioavailability in inner ear, some preclinical studies suggest repeating the USMB treatment after a renewal of MBs between each US exposure [99, 103]. Indeed, two USMB treatments significantly increased the concentration of biotin-FITC in the perilymph compared to control group [103]. Another study demonstrated that five consecutive USMB treatments may sustain a more pronounced permeability change in the RWM than three treatments [99].

While successful delivery of therapeutic molecules to the perilymph is the main objective, it is also important that such molecules are released all along the cochlea, from the basal turn and to the apex following the baso-apical gradient [187]. This gradient is due to minimal flow in the cochlear fluids [54] and also to perilymph washout direction [188]. The degree of intracellular uptake of therapeutic molecules decreases along the baso-apical gradient in the cochlea, with minimal uptake at the cochlear apex [103]. Transtympanic injection of gadolinium or nanoparticles revealed the presence of this gradient [189-191]. Recently, the existence of this gradient was observed after gentamicin delivery using USMB, with a decrease in gentamicin uptake from basal turn to apex of the cochlea [103].

3.2 When can we deliver molecules in the human inner ear?

While USMB is under clinical investigation for delivery of therapeutic molecules for the treatment of cancers, brain and cardiovascular disorders, there is no present clinical trial for the treatment of hearing disorders [192, 193]. Before application to humans, a US probe adapted to the morphology of the external auditory canal must be developed and validated. Thus, we can consider a possible transtympanic injection of MBs and the desired therapeutic molecules before positioning the US probe head in contact with the tympanic membrane. The whole procedure would be less invasive than in animal trials. The procedure could even be performed

under local anesthetic during a conventional consultation without hospitalization. The US parameters needs to be adjusted depending on the drug to be delivered.

4. Conclusion

Sonoporation is an innovative and promising US modality for efficient and safe delivery of therapeutic molecules in the inner ear to treat hearing disorders. In vitro and preclinical studies have thus far reported encouraging results in terms of both efficacy and safety of this modality. These promising data suggest that sonoporation may revolutionize the management of inner ear pathologies in the coming years. Further investigations are required to develop standardized protocol for a clinical application of this drug delivery method.

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Figure captions

Graphical abstract: Inner ear delivery and sonoporation

Legends: CN: Cochlear nerve; HL: Hearing loss; MBs: Microbubbles; SM: Scala media; ST: Scala tympani; SV: Scala vestibuli; US: Ultrasound; USMB: Ultrasound Microbubbles.

Figure 1: Auditory system. A: Anatomy of a right human ear. B: Cross-sectional view of the cochlea. C: View of the middle ear through the external auditory canal in a human cadaver.

Legends: 1. External ear pinna; 2. Temporal muscle; 3. Temporal meninge; 4. Temporal bone; 5. Vestibule; 6. Endolymphatic sac; 7. Cochlear nerve; 8. Cochlea; 9. Auditory tube; 10. Round window membrane; 11. Oval window membrane; 12. Ossicular chain; 13. Tympanic membrane; 14. External auditory canal; 15. Scala vestibuli; 16. Scala media; 17. Organ of Corti; 18. Scala tympani; 19. Long process of the incus; 20. Malleus handle; 21. Tympanic membrane remnant; 22. Promontory, corresponding to the first turn of the cochlea; 23. Pyramid; 24. Stapedial muscle; 25. Stapes.

Figure 2: Various approaches are known for delivering therapeutic molecules to the inner ear. These include injections directly into the inner ear, with opening of the otic capsule: canalostomy (1), opening of the utricle (2), and cochleostomy (3). Injections through the oval window membrane via the annular ligament (4) and the round window (5) are also studied, as well as injection into the endolymphatic sac (6). (7) shows a needle for transtympanic injection at the postero-inferior part of the tympanic membrane. The labyrinthine artery (8) enables therapeutic molecules to be diffused by the systemic route.

Figure 3: PRISMA Flow Diagram. The PRISMA diagram details the search and selection process applied during the review.

Table 1: Studies of sonoporation in the inner ear

Legends: ABR: Auditory brainstem response; AAV: Adeno-associated virus; CS-AuNPs: Chitosan-coated gold nanoparticles; DPOEA: Distortion product otoacoustic emissions; ELISA: Enzyme-linked immuno-sorbent assay; IGF-1: Insulin-like growth factor-1; MBs: Microbubbles; NA: Not available; PCR: Polymerase chain reaction; P407: Poloxamer 407; RWM: Round window membrane; T°C: Temperature in degrees Celsius.

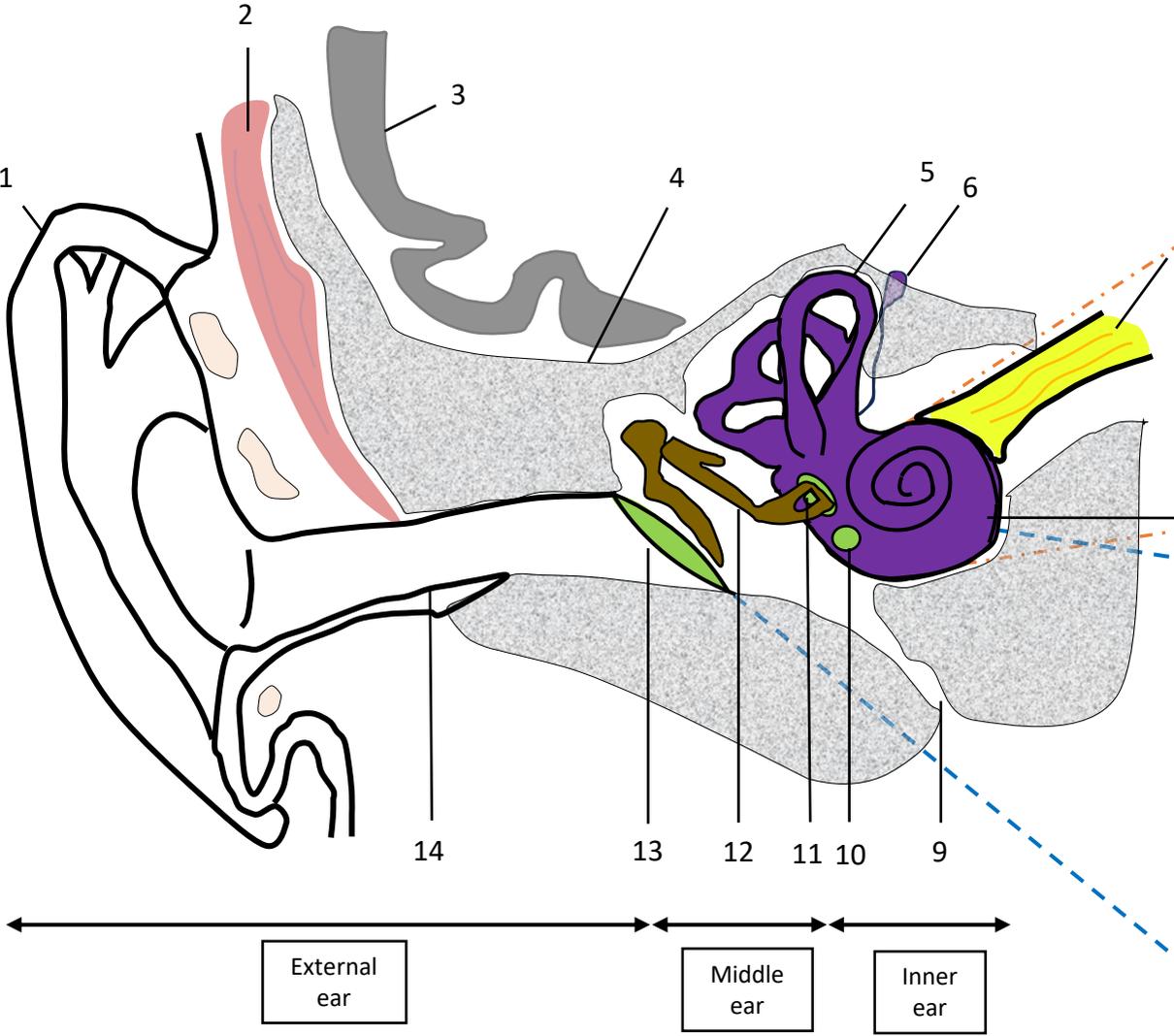
Table 2: In vitro sonoporation in the inner ear (n=5)

Legends: DNA: Deoxyribonucleic acid; CSAuNPs: Chitosan-coated gold nanoparticles; FITC: Fluorescein isothiocyanate; MBs: Microbubbles; IGF-1: Insulin-like growth factor 1; ISATA: Intensity spatial average, temporal average; ISPTA: Intensity spatial peak, temporal average; NA: Not available; P407: Poloxamer 407; US: Ultrasound.

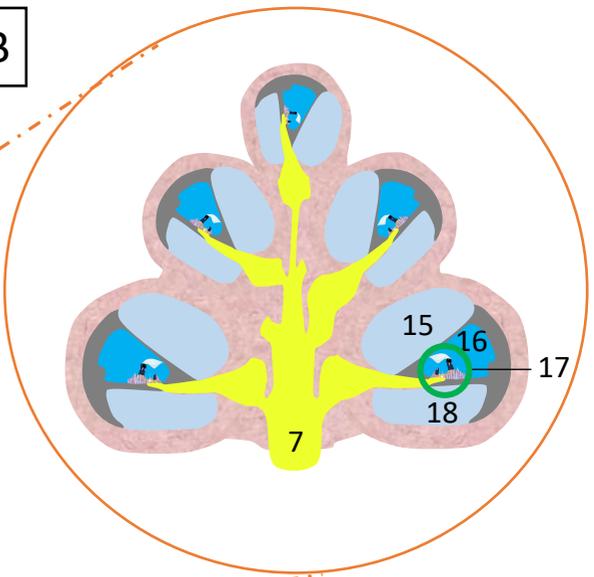
Table 3: In vivo sonoporation in the inner ear (n=11)

Legends: CSAuNPs: Chitosan-coated gold nanoparticles; FITC: Fluorescein isothiocyanate; MBs: Microbubbles; IGF-1: Insulin-like growth Factor 1; ISATA: Intensity spatial average, temporal average; ISPTA: Intensity spatial peak, temporal average; NA: Not available; P407: Poloxamer 407; US: Ultrasound.

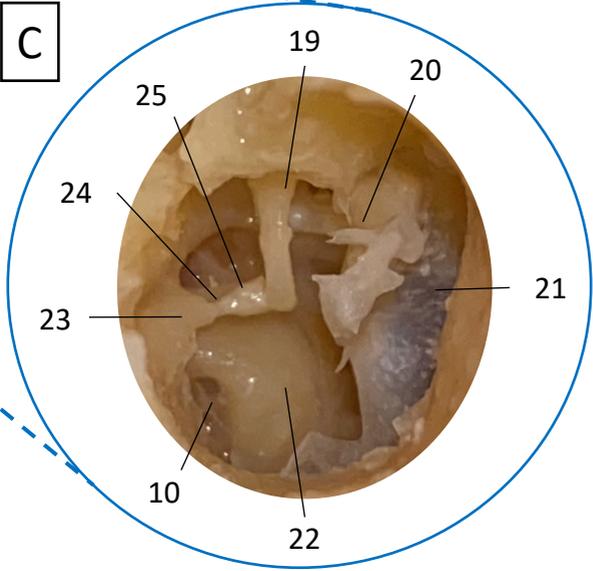
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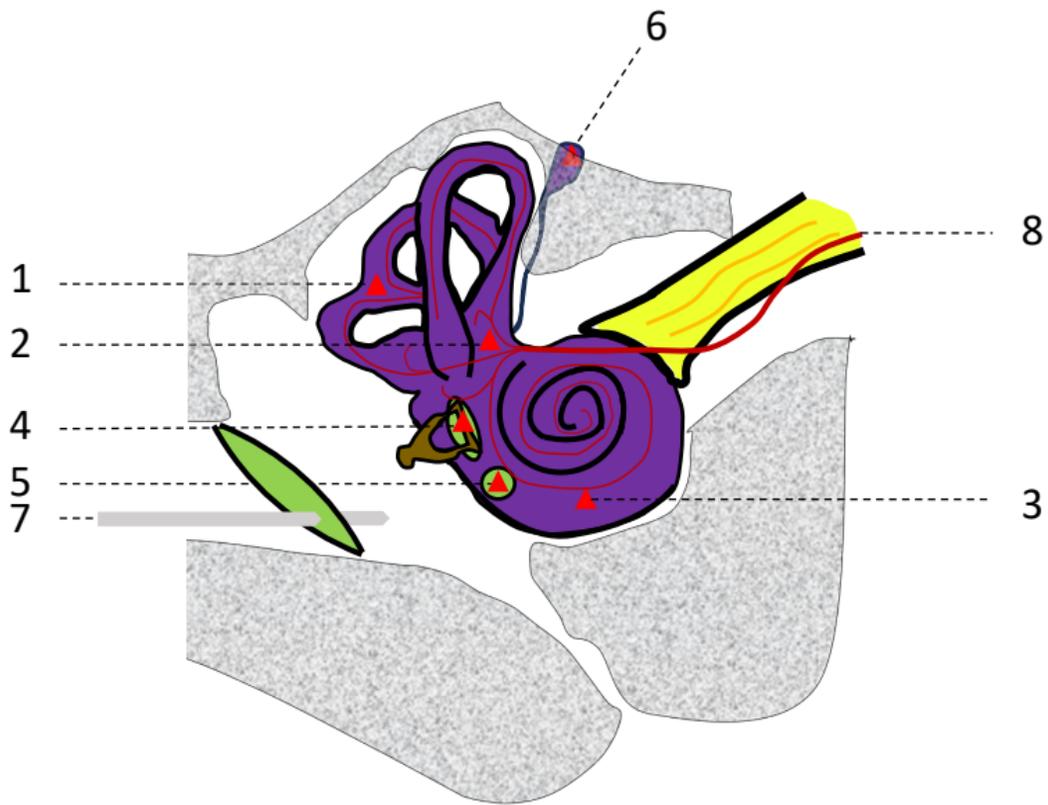


B



C





Identification

Screening

Eligibility

Included

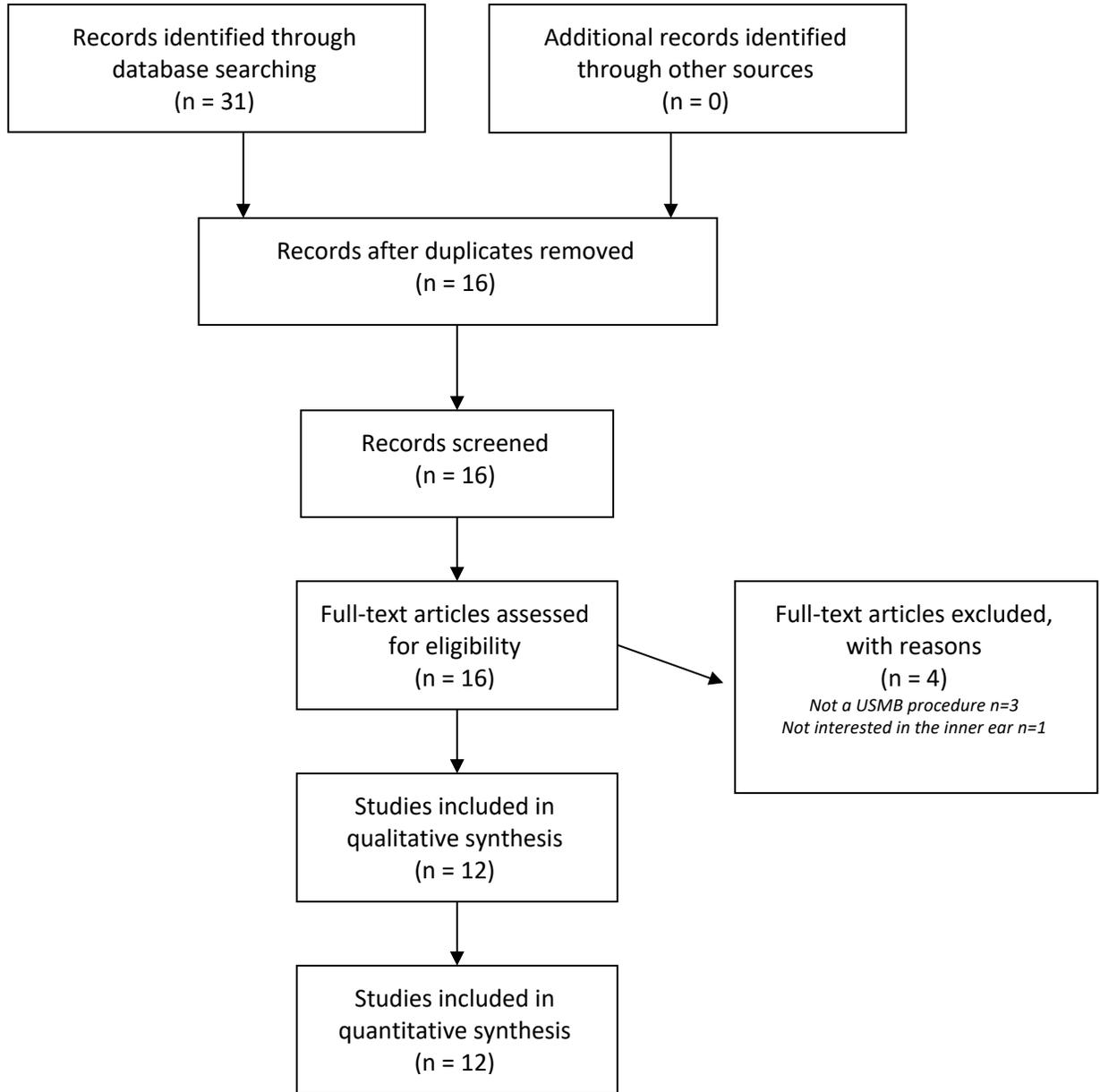


Table 1: Studies of sonoporation in the inner ear

References	Study design	Animals (N) In vitro	Objective	Parameters	Main findings
Kerneis et al. (2023) [93]	RCT	Sheep (6)	To assess the safety of sonoporation of inner ear in large animal	ABR, T°C, Metabolic investigations	No ototoxicity found.
Liao et al. (2022) [94]	RCT	Guinea pigs (NA) In vitro	To assess the efficacy of sonoporation using dual frequency	ABR, T°C, Microscopic investigations, ELISA	Dual-frequency US treatment increased efficacy of drug delivery by up to 60%, compared to single-frequency US treatment.
Liao et al. (2021) [95]	RCT	Guinea pigs (NA) In vitro	To study potential US-mediated P407-based MBs cavitation for inner ear drug delivery	ABR, DPOAE, Microscopic investigations, ELISA	US-mediated P407-MBs gel in the middle ear cavity enhanced the delivery of drugs into the inner ear.
Lin et al. (2021) [96]	RCT	Mice (68) In vitro	To explore the use of CS-AuNPs to increase drug delivery in the inner ear	ABR, DPOAE, Microscopic investigations	USMB enhanced the delivery of CS-AuNPs to the inner ear
Lin et al. (2021) [97]	RCT	Guinea pigs (47)	To assess the therapeutic efficacy of the delivery of IGF-1 using sonoporation to the inner ear after noise exposure	ABR, Microscopic investigations, ELISA, PCR	IGF-1 delivery with sonoporation was more effective for hearing loss than without sonoporation.
Liao et al. (2020) [98]	RCT	Guinea pigs (30) In vitro	To compare the efficacy of drug delivery into the inner ear using both transcanal or transcranial approaches	ABR, T°C, Microscopic investigations	The transcanal approach induced a 280% increase in drug delivery, compared to a 159% increase with the transcranial approach.
Lin et al. (2020) [99]	RCT	Guinea pigs (58)	To permeabilize RWM	ABR, DPOAE, T°C, Microscopic investigations	Permeability of RWM was significantly enhanced with sonoporation
Zhang et al. (2020) [100]	RCT	Guinea pigs (27)	To transduce gene through RWM using AAV	ABR, Microscopic investigations	Gene transduction was possible.
Shih et al. (2019) [101]	RCT	Guinea pigs (42)	To prevent the noise-induced effects using sonoporation-mediated delivery of dexamethasone	ABR, Microscopic investigations, ELISA	Dexamethasone delivery with sonoporation showed significant protection against noise exposure.

Liao et al. (2014) [102]	Observational	In vitro	To improve gene transfection by sonoporation	Microscopic investigations	MBs of different sizes can be produced by adjusting the concentration of albumin or dextrose alone or the combined albumin and dextrose mixture to improve gene transfection.
Shih et al. (2013) [103]	RCT	Guinea pigs (60)	To deliver drug to the inner ear with sonoporation	ABR, Microscopic investigations	Sonoporation enhanced drug delivery through the RWM.
Liao et al. (2012) [104]	RCT	Guinea pigs (NA)	To deliver drug to the inner ear with sonoporation	ABR, Microscopic investigations	Sonoporation improved drug delivery through the RWM.

ABR: Auditory brainstem response; AAV: Adeno-associated virus; CS-AuNPs: Chitosan-coated gold nanoparticles; DPOEA: Distortion product otoacoustic emissions; ELISA: Enzyme-linked immuno-sorbent assay; IGF-1: Insulin-like growth factor-1; MBs: Microbubbles; NA: Not available; PCR: Polymerase chain reaction; P407: Poloxamer 407; RCT: Randomized control trial; RWM: Round window membrane; T°C: Temperature in degrees Celsius.

Table 2 : In vitro sonoporation in the inner ear (n=5)

References	Drug	Microbubbles	Resonance frequency of the microbubbles	Microbubbles concentration	US parameters			
					Frequency	Acoustic pressure/ intensity	Duty cycle	Total insonation time in minutes
Liao et al. (2022) [94]	IGF-1	Albumin-shelled	NA	1.2×10^7 MBs/mL	1 MHz/ 666 kHz + 1MHz	0.426 W/cm ² (I _{SPTA})	50%	3
Liao et al. (2021) [95]	Dexamethasone P407 + Biotin-FITC	Albumin-shelled	NA	4.2×10^7 MBs/mL	1 MHz	3 W/cm ² (I _{SATA})	50%	1
Lin et al. (2021) [96]	CSAuNPs	SonoVue	2,5 MHz	2×10^8 MBs/mL	1 MHz	1, 2, 3 W/cm ² (I _{SATA})	NA	3x1
Liao et al. (2020) [98]	Biotin-FITC	Albumin-shelled	NA	1.4×10^7 MBs/mL	1 MHz	1, 2, 3, 4 W/cm ² (I _{SATA})	50%	Transcanal : 1 Transcranial : 3
Liao et al. (2014) [102]	Plasmid DNA	Albumin-shelled	NA	10-18 $\times 10^8$ MBs/mL for albumin concentration; 4-16 $\times 10^8$ MBs/mL for dextrose concentration	3.185 MHz	0.2-0.84 W/cm ² (I _{SPTA})	50%	2

DNA: Deoxyribonucleic acid; CSAuNPs: Chitosan-coated gold nanoparticles; FITC: Fluorescein isothiocyanate; MBs: Microbubbles; IGF-1: Insulin-like growth factor 1; I_{SATA}: Intensity spatial average, temporal average; I_{SPTA}: Intensity spatial peak, temporal average; NA: Not available; P407: Poloxamer 407; US: Ultrasound.

Table 3: In vivo sonoporation in the inner ear (n=11)

References	Drug	Microbubbles	Resonance frequency of the microbubbles	Microbubbles concentration	US parameters			
					Frequency	Acoustic pressure/ intensity	Duty cycle	Total insonation time in minutes
Kerneis et al. (2023) [93]	Free MBs	Vevo MicroMarker	4 MHz	2×10^8 MBs/mL	1.1MHz	300 kPa	40%	3 x 1
Liao et al. (2022) [94]	IGF-1	Albumin-shelled	NA	1.2×10^7 MBs/mL	1 MHz/ 666 kHz + 1MHz	$0.213 \text{ W/cm}^2 (I_{SPTA})$	50%	3
Liao et al. (2021) [95]	Dexamethasone P407	Albumin-shelled	NA	4.2×10^7 MBs/mL	1 MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	1
Lin et al. (2021) [96]	CSAuNPs	SonoVue	2.5 MHz	$2\text{-}5 \times 10^8$ MBs/mL	1 MHz	$2 \text{ W/cm}^2 (I_{SATA})$	50%	3 x 1
Lin et al. (2021) [97]	IGF-1	SonoVue	2.5 MHz	$2\text{-}5 \times 10^8$ MBs/mL	1 MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	1
Liao et al. (2020) [98]	Gentamicin	Albumin-shelled	NA	1.40×10^7 MBs/mL	1 MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	Transcanal: 1 Transcranial: 3
Lin et al. (2020) [99]	Biotin-FITC	SonoVue	2.5 MHz	$2\text{-}5 \times 10^8$ MBs/mL	1MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	3 or 5
Zhang et al. (2020) [100]	Gene transfection	Definity	10 MHz	$1\text{-}5 \times 10^8$ MBs/mL	1.7 MHz	350-400 kPa	NA	5
Shih et al. (2019) [101]	Dexamethasone	SonoVue / Albumin-shelled	2.5 MHz / NA	2.9×10^8 MBs/mL	0.5, 1, 3, 5 MHz	$3 \text{ W/cm}^2 (I_{SPATA}$ for 0.5 and 1 MHz and I_{SATA} for 3 and 5 MHz)	50%	3 x 1
Shih et al. (2013) [103]	Biotin-FITC or gentamicin	Albumin-shelled	NA	4×10^9 MBs/mL	1 MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	1 x 1 or 2 x 1
Liao et al. (2012) [104]	Biotin-FITC	Albumin-shelled	NA	4×10^9 MBs/mL	1 MHz	$3 \text{ W/cm}^2 (I_{SATA})$	50%	1

CSAuNPs: Chitosan-coated gold nanoparticles; FITC: Fluorescein isothiocyanate; MBs: Microbubbles; IGF-1: Insulin-like growth factor 1; I_{SATA} : Intensity spatial average, temporal average; I_{SPTA} : Intensity spatial peak, temporal average; NA: Not available; P407: Poloxamer 407; US: Ultrasound.