The ototoxic effect of locally applied kanamycin and furosemide in guinea pigs

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ABSTRACT

Background: Hearing impairment is a growing social and economic issue. New technical or biological approaches aiming hearing rehabilitation or regeneration require animal testing. Therefore, a reproducible and safe model for hearing-impaired animals is essential.

New method: Intratympanic injection of kanamycin and furosemide was administered for BFA bunt pigmented guinea pigs for either 1 or 2 h. Hearing loss was regularly measured with compound action potential response to click and tone burst stimuli for up to 26 weeks. Hair cell loss and the density of spiral ganglion neurons were histologically analyzed.

Results: One week after the exposure, complete hearing loss was observed in 34 ears from the 36 ears treated for 2 h and remained stable during the follow-up. Histology revealed near complete hair cell loss and secondary degeneration of spiral ganglion neurons.

Comparison with existing methods: Animal deafening is usually achieved by systemic application of aminoglycoside antibiotics or chemotherapy drugs, although side effects such as nephrotoxicity may occur which can be avoided by local application. With our procedure, unilateral hearing loss model can also be established.

Conclusions: The single intratympanic application of a solution of 200 mg/ml kanamycin and 50 mg/ml furosemide is a stable and reliable deafening method.

1. Introduction

Due to the aging population, hearing impairment becomes a growing social and economic issue worldwide (Wilson et al., 2019). As the most common cause of sensorineural hearing worldwide is cochlear hair cell (HC) damage and no spontaneous hair cell regeneration occurs in mammals, treatment for hearing rehabilitation is currently restricted to hearing aids and cochlear implants (Davis, 1983). New approaches, such as the induced regeneration of hair cell recipients or delivery of neurotrophic factors, have attracted increased attention, but these strategies first require animal testing. Therefore, establishing a reproducible and safe hearing-impaired animal model is essential.

The most common method for this purpose is the systemic application of aminoglycoside antibiotics or chemotherapy drugs. They induce an irreversible damage mainly to HCs (Hawkins, 1976). Kanamycin alone requires repetitive administrations to achieve permanent hearing loss, but coadministration with a loop-diuretic drug enhances its effect (Song et al., 1998; West et al., 1973). Currently, the administration of subcutaneous kanamycin (400 mg/kg) with intravenous furosemide (100 mg/kg) is a routinely used method for guinea pigs, resulting in a reliable threshold shift (Shepherd et al., 2005; Vennel et al., 2007). Unfortunately, side effects such as the nephrotoxicity of aminoglycoside antibiotics are also enhanced with the systemic coadministration of loop diuretics, both in animals and humans (Adelman et al., 1979; Rodriguez Salguero and Gonzalez Núñez, 2016; Smith and Lietman, 1983). In the past, local administration of kanamycin was also used to avoid systemic side effects. Kellerhals applied a single injection of kanamycin through
the tympanic membrane of guinea pigs (Kellerhals, 1967). Hashimoto et al., (2007) achieved a reliable loss of cochlear HCs perfusing a high concentration of kanamycin (172.5 or 345 mg/ml) through a microcatheter with an infusion rate of 0.1 ml/h for 1 and 2 h. The intratympanic application of kanamycin and furosemide has been studied in rats, where a gelatin sponge soaked in 50 µl of a mixture of kanamycin and furosemide (concentration of 200 mg/ml kanamycin and 50 mg/ml furosemide) was placed into the round window niche (Murillo-Cuesta et al., 2009).

Our aim was to develop a reproducible guinea pig animal model utilizing a single intratympanic injection resulting in near-complete HC loss. For that purpose, guinea pigs were injected intratympanically with a combination of kanamycin and furosemide.

2. Materials and methods

2.1. Animals and Experimental Design

Sixty-five ears of pigmented guinea pigs (n = 40; BFA bunt; 310–560 g) were included in this study. The animals were kept in standard laboratory conditions and had free access to food and water. All experimental procedures were approved by the Committee for Animal Experiments of the Regional Council (Regierungspräsidium; Ref. No.: HN 2/11) of Tübingen.

The animals were divided into 3 experimental groups. Ears were treated with the deafening solution for 1 or 2 h in Group “1 h” and Group “2 h”, respectively, whereas no solution was applied to the control group (Table 1). For histological analysis, the animals were sacrificed at different times, at 5, 14 and 26 weeks after deafening. No histological analysis after 1 week was performed.

2.2. Surgical procedure

All surgical and deafening procedures and hearing measurements were performed under general anesthesia. The animals were anesthetized with an intramuscular injection of fentanyl citrate (0.025 mg/kg; Fentadon, Eurovet Animal Health), Southampton, UK, midazolam (0.2 mg/kg; Midazolam-Ratiopharm, Ratiopharm, Ulm, Germany) and medetomidine hydrochloride (1 mg/kg; Sedator, Eurovet Animal Health). The animals were placed on heating pads at 37 °C during anesthesia.

The bulla was opened via a retroauricular approach. The bare tip of an insulated gold wire (Goodfellow, London, UK) was placed in the organ of Corti (OC). Immunostaining with myosin VIIA (Myo7A, rabbit, 1:400, Axxora LLC, Farmingdale, NY, USA) was published previously (Bako et al., 2015). After orientation, 7 µm sections were cut out. For quantification of the remaining hair cells, every 5th section was stained with Epoxy Tissue Stain (ETS, Electron Microscopy Sciences, PA, USA) for 2 min. Hair cells from at least 10 midmodiolar sections (spaced 35 µm) were analyzed and counted with a Zeiss Axioscope 2 (Zeiss, Oberkochen, Germany) at 40x magnification from the different cochlear half-turns, according to the shape and position of the cells within the organ of Corti (OC). Immunostaining with myosin VIIA (Myo7A, rabbit, 1:400, Axxora LLC, Farmingdale, NY, USA) was performed in the neighboring sections to verify the results from ETS staining. The definition of Van Ruijven et al. wa used to define the cochlear turns (Fig. 3A). Half-turns apical to a1 were defined as half-turns a+ (Van Ruijven et al., 2005). Outer and inner hair cell loss (OHC, IHC) were analyzed in 10 cochleae of Group 1 h and Group 2 h, respectively. A value of 100% indicated that all 3 outer hair cells could be observed in the sections analyzed.

Spiral ganglion neuron (SGN) packing density was measured to determine the effect of the deafening procedure on the SGNs. Therefore, midmodiolar ETS-stained sections were analyzed. The sectional area of the cells within the organ of Corti (OC) was measured using Zen 2012 Software. The SGN density was calculated in cells/mm².

2.4. Hearing measurements

Compound action potential (CAP) measurements were performed before the deafening procedure, immediately after the exposure, and 1, 3, 5, 9, 14, 22 and 26 weeks later. For the reference and grounding electrodes, a silver wire electrode (0.25 mm, Goodfellow) was subcutaneously inserted into the back of the animals and above the bregma.

The measurements were made in a sound-proof chamber. For stimulation, either clicks (duration: 100 ms, rise/fall: 1 ms) or tone-burst stimuli (duration: 3 ms, rise/fall: 1 ms) were presented. Stimuli were generated with custom-made software. Recordings were bandpass filtered (0.2 kHz and 5 kHz) and amplified to 60 dB or 80 dB. For input/output (I/O) measurements, tone-burst stimuli were used in 3 dB steps from 0 to 108 dB SPL at 14 frequencies between 0.5 and 45.2 kHz (2 frequencies/octave). The threshold for the tone-burst stimulus was measured between 0.5 kHz and 45.2 kHz at a resolution of 8 steps/octave (53 frequencies), utilizing a Wald test and a Pest algorithm to search for the threshold (Gummer et al., 1987; Müller et al., 2005). For a simplified visualization of the threshold, the pure threshold average (PTA) was defined as the average threshold of the 53 frequencies tested.

2.5. Histological analysis

After the last hearing measurement, the animals were sacrificed, and the cochleae were removed for histological analysis. After intracardial injection of 0.5 ml of T61 (Intervet Deutschland, Unterschleißheim, Germany) under deep anesthesia, each animal was perfused with ca. 50 ml of Ringer’s solution, followed by 200 ml of 4% formaldehyde (Roti-HistoFix, Roth, Karlsruhe, Germany). The animals were then decapitated, and the cochleae were harvested, perfused with 4% formaldehyde through the round and oval windows, postfixed for 2 h in 4% formaldehyde, and stored in 1% formaldehyde. The cochleae were decalcified with 0.2 M EDTA (Sigma-Aldrich) for 4–6 days. The cochleae were embedded in a methyl methacrylate-based resin embedding system (Technovit 9100, Heraeus Kurzer). The detailed embedding protocol was published previously (Bako et al., 2015). After orientation, 7 µm sections were cut out. For quantification of the remaining hair cells, every 5th section was stained with Epoxy Tissue Stain (ETS, Electron Microscopy Sciences, PA, USA) for 2 min. Hair cells from at least 10 midmodiolar sections (spaced 35 µm) were analyzed and counted with a Zeiss Axioscope 2 (Zeiss, Oberkochen, Germany) at 40x magnification from the different cochlear half-turns, according to the shape and position of the cells within the organ of Corti (OC). Immunostaining with myosin VIIA (Myo7A, rabbit, 1:400, Axxora LLC, Farmingdale, NY, USA) was performed in the neighboring sections to verify the results from ETS staining. The definition of Van Ruijven et al. was used to define the cochlear turns (Fig. 3A). Half-turns apical to a1 were defined as half-turns a+ (Van Ruijven et al., 2005). Outer and inner hair cell loss (OHC, IHC) were analyzed in 10 cochleae of Group 1 h and Group 2 h, respectively. A value of 100% indicated that all 3 outer hair cells could be observed in the sections analyzed.

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Table 1

| Group | Exposure Time (h) | No. of Ears | Audiological Follow-up | 1 Week | 5 Weeks | 14 Weeks | 26 Weeks |
|-------|------------------|-------------|------------------------|--------|---------|----------|---------|
| 1 h   | 1                | 21          | 8                      | 4      | 3       | 6        |
| 2 h   | 2                | 36          | 22                     | 11     | 3       |
| Control | –              | 8           | 1                      | 2      | 1       | 4        |

The statistical analysis was performed with SPSS v22 software (IBM Corporation). An independent samples t-test was used to compare the SGN densities of the different ears. Statistical significance was defined if
3. Results

3.1. Audiological results

3.1.1. Threshold and I/O measurements

For analysis of the effect of long-term electrode implantation, the bullae in the control group were only opened for electrode implantation. The measurements of 1 ear from the control group are shown in Fig. 1A, C and E. The threshold values below 3 kHz were higher on the day of electrode placement than those 1, 9 and 26 weeks later. On Day 0, the highest threshold (71 dB SPL) was observed at 1.54 kHz and was then lowered to 50, 46 and 45 dB SPL at 1, 9 and 26 weeks later, respectively (Fig. 1A). A deterioration in the threshold between 5.1 and 9.5 kHz could be detected in the measurements 26 weeks after placing the electrode, with a maximum SPL of 42 dB at 7.33 kHz. The threshold values at frequencies higher than 9.5 kHz were similar in all measurements.

Alterations of the CAP-wave amplitudes (I/O measurement) were visualized at 4 different times at 1 and 16 kHz (Fig. 1C, E). Compared to later measurements, the amplitudes were reduced at both frequencies in the measurements of Day 0. An elevated threshold could only be observed at 1 kHz (57 dB SPL). No difference in amplitude or threshold during the follow-up was found. In terms of both hearing threshold and CAP amplitudes, no effect of the intervention (opening the bulla and placing the electrode) itself was detected; hearing remained stable for the complete experimental period (26 weeks).

The effects of the deafening procedure on the threshold and CAP amplitude were demonstrated at different dates on an ear from Group 2 h. The audiogram before the intervention served as a reference and proved the pretreatment of the normal hearing of the ear (Fig. 1B).
Immediately after deafening, hearing loss was observable at all frequencies, ranging from 31 dB (at 4.3 kHz) to 79 dB (at 24.7 kHz), with a tendency toward more severe damage at higher frequencies. One week after deafening, no CAP response could be detected. Therefore, the values of the calibration curve (the possible highest sound pressure our system could present) represent the “hearing threshold” of these measurements. Since these values were higher than 90 dB SPL at all frequencies, the ears were defined as deaf ears. Hearing loss remained stable during the complete experimental period; no hearing improvement could be observed after 9 or 26 weeks.

The immediate effect of the procedure could be observed on the CAP waves, reducing the amplitude value and shifting the threshold. At 1 kHz, the maximum amplitude before deafening was 57 µV, while the threshold was at 45 dB SPL. Immediately after the procedure, the threshold increased to 84 dB SPL, with a maximum amplitude of 7.7 µV (Fig. 1D).

At 16 kHz, the threshold before deafening was 36 dB SPL, with a maximum amplitude of 64 µV. After the procedure, the threshold shifted to 84 dB SPL, with a maximum amplitude of 10.3 µV (Fig. 1F). No CAP responses could be detected by later measurements.

Within 1 week after ototoxic exposure, significant hearing loss appeared. One week after the intervention, no response to any acoustic stimulus could be detected by measurements. The hearing measurements after 9 and 26 weeks proved that the effect remained constant, without any reversible component during our follow-up. The ears were regarded as deaf ears 1 week after the procedure.

3.1.2. PTA values after 1 week

As hearing values after 1 week were found to be stable during the follow-up period, the percentage of successful deafening is shown on the PTA values for all ears 1 week after the procedure (Fig. 2).

An average PTA of all ears recorded immediately after electrode placement (before deafening) served as a reference (41.6 dB SPL ± 9.3 dB SPL). Ears in different experimental groups with no detectable CAP response were regarded as deaf ears.

Among the animals treated for 1 h, 17 out of 21 ears were deaf after 1 week (80.1%). Among the 4 non-deafened ears, PTA values of 72, 73, 96, and 104 dB SPL could be measured. Among the animals treated for 2 h, 34 of the 36 ears (94.4%) were regarded as deaf after 1 week. Only moderate hearing loss was achieved (PTA values: 66, 73 dB SPL) in 2 ears. These ears represent 2 ears of the same animal. No hearing loss was observed in the control group.

![PTA values after 1 week](image)

**Fig. 2.** PTA values of all ears 1 week after the surgery in the different experimental groups. The black dotted line represents the average PTA value of all ears from the measurement immediately after placing the electrode; the area around it represents the SD of these PTA values. Each dot above the black line represents an ear in different experimental groups with no detectable CAP response, which are regarded as deaf ears. The percentage of deaf ears in each group is shown.

3.2. Histological results

3.2.1. Hair cell loss

As a background of the threshold deterioration, morphological changes of the OC were observed (Fig. 3). As an example, OC of the 2nd basal turn (b2) of an ear from the control group and Group 2 h demonstrated by ETS and immunohistochemical staining (Fig. 3B and C, D and E, respectively). Both animals were sacrificed after the experimental period of 26 weeks. The normal structure of the OC was observed in the control ears with Myo7A staining of both the IHCs and OHCs, and NF-200 staining of the peripheral processes of the SGNs heading toward the IHCs could also be observed. A flat layer of epithelium was observed in the deafened ear, without any sign of the structure of the former OC (Fig. 3D). No immunolabeling with Myo7A or NF-200 was observed (Fig. 3E). The permanent changes in the OC could be justified.

The survival of the HCs of the deaf ears from the Groups 1 h and 2 h as well as of ears of the Control Group were analyzed (Fig. 3F and G). Regarding the OHCs, no survival could be observed in the basal (b1 and b2) and middle half-turns (m1 and m2) in either of the deafened groups (Fig. 3F). The ratio of surviving OHCs remained under 10% (9%, 2%, 8%, 4%) in all apical 4 half-turns (a1, a2, a3, and h) in Group 1 h. The percentages of surviving OHCs were 1%, 4%, 11% and 39% in a1, a2, a3 and the helicotrema, respectively, in Group 2 h. In the Control Group, the rate of the clearly detectable OHCs was above 91% in all half-turns. The lowest rate (91.5%) was found to be in the a2 half-turn. Regarding the IHCs, no survival could be detected in either of the deafened groups in the b1 half-turn. Within Group 1 h, IHC survival was 5% in the b2 half-turn, 1% in m1, 3% in m2, 2% in a1, 0% in a2 and 2% in a3. Within Group 2 h, a percentage of 1%, 3%, 1% and 10% of the IHCs survived in the half-turns b2, a2, a3 and the helicotrema, respectively, although no survival in m1, m2 and a1 was detected. Survival rate of the IHCs of the control ears was equal or above 98% in all cochlear half-turns with the lowest rate in the b2 half-turn.

3.2.2. Degeneration of the spiral ganglion neurons

The degeneration of SGNs begins after hair cell loss and can be characterized by a reduction in the number of SGNs. The animals in Group 1 h were sacrificed at different times to analyze the changes in SGN density over time. Demonstrating the SGN density reduction over time, the cross-sections of Rosenthal’s canal of the cochlear b2 half-turns of different ears of Group 1 h and an ear of the control group (referring to Rosenthal’s canal of a normal hearing animal) are shown in Fig. 4A-D. Neurons were located tightly and filled Rosenthal’s canal in the cross-section of Rosenthal’s canal of one ear from the control group, while smaller groups of cells were separated by processes. A reduction in the number of SGNs could already be observed in Rosenthal’s canal section of the b2 half-turns in deaf ears 5 weeks after deafening. The peripherally located cells of the canals were mainly reduced with a relatively intact part in the middle (Fig. 4B). A further decrease in neurons was detected, as shown in Fig. 4C and D. For analysis of the SGN density deterioration over time, the densities of deaf ears of the animals in Group 1 h, sacrificed 5, 14 and 26 weeks after deafening, were compared to the densities of the normal hearing animals (the control group), sacrificed after a follow-up of 26 weeks (Fig. 4E). The average SGN densities of the half-turns among the normal hearing animals were found to be between 1458.81 cells/mm² (in the half-turn b1) and 1650.58 cells/mm² (in m2), with SD between 164.19 (in m1) and 288.78 (in a1), except in the half-turns a2 (1000.54 cells/mm²; SD: 260.77). Five weeks after the deafening procedure, a significant reduction in SGN density could be observed in all half-turns. The densities were found to be between 542.37 cells/mm² (in b1) and 822.69 cells/mm² (in m1). The SGN densities were significantly reduced 14 weeks after deafening compared to the results of all half-turns after 5 weeks. The lowest density was found in half-turn a + (221.12 cells/mm²; SD: 58.82), and the highest density was observed in half-turn m2 (440.25 cells/mm²; SD: 133.9). No significant difference could be found.
between the SGN density values of the ears sacrificed at 14 and 26 weeks after deafening, except in the m2 half-turn, where values after 26 weeks were higher than those after 14 weeks. These results show a reduction in SGNs over time, which was already proven to be significant after 5 weeks.

Analyzing the ratio of the average SGN densities of the deafened ears sacrificed after 5 weeks to the average of the control group, a baso-apical gradient could be witnessed. After 5 weeks only 38% of the SGNs are present in b1. The ratio is increasing toward the apex almost continuously (b2: 42%; m1: 53%; m2: 44%; a1: 45%, a+: 64%).

To compare the effect of the different exposure time on the SGN density, ears from both groups sacrificed after 14 weeks were analyzed (Group 1 h, n = 3; Group 2 h, n = 6). No significant differences could be found in most of the half-turns, however significantly higher density was measured in the b1 cochlear half-turn in Group 2 h.

3.3. The relation between audiological and histological results

High-frequency permanent hearing loss and the corresponding HC loss and lower SGN packing density demonstrate the tight relations between audiological and histological results. The results of a partially deafened ear from Group 2 h are shown in Fig. 5. The animal was sacrificed 14 weeks after the deafening procedure. Fig. 5A shows the audiograms of the ear before and 14 weeks after the deafening procedure. The audiogram before deafening showed the same values as in Fig. 1A and B. A maximum hearing loss of 14 dB (at 3.36 kHz) was observed in the audiogram after 14 weeks at frequencies below 4 kHz. A considerable loss of hearing threshold was observed above 4 kHz, and no further CAP waves could be detected from 20.7 kHz on, with the values of the audiogram corresponding to the values of the calibration curve. Fig. 5B shows the ratio of the surviving HCs after histological analysis of the ears. No surviving HCs were visible in the b1 half-turn. Forty-two percent of the outer and 94% of the IHCs survived in the b2 half-turn. A survival rate of 93–100% of the OHCs was detected in the m1 half-turn, while all the IHCs showed complete survival (100%). The SGN density values of the ears in the different half-turns were also analyzed (Fig. 5C). A reduced density (604.76 ells/mm$^2$) could be detected in b1, namely, 41% of the normal values, as shown in Fig. 4E, although this value rose to 231% of the SGN density of the average of deaf ears (compared to the values of the deaf ears sacrificed after 14 weeks, shown in Fig. 4E). The density value in the b2 half-turn almost reached the normal level (1521.3 cells/mm$^2$, compared to 1610.53 cells/mm$^2$), and apically from there, the values were similar to normal. Incomplete hearing loss could only be achieved with this animal, resulting in HC loss mainly only in the basal turns. Due to the surviving HCs in the more apical turns, no degeneration of SGNs in the corresponding half-turns was observed.

3.4. Surgical difficulties and complications

The level of the deafening solution showed a constant decrease during the exposure of 2 animals. In these cases, refilling of the bulla was
Fig. 4. A. ETS-staining of the cross-section of the Rosenthal’s canal of the b2 half-turn of a normal hearing ear of the Control Group sacrificed after 26 weeks. Neurons are located tightly and they fill the Rosenthal’s canal. B. ETS-staining of the Rosenthal’s canal of the b2 half-turn of a deaf ear of Group 1 h, sacrificed after 5 weeks. Reduction of the number of SGNs is visible mainly in the peripheral part of the canal. C. ETS-staining of the Rosenthal’s canal of the b2 half-turn of a deaf ear of Group 1 h, sacrificed after a follow-up of 14 weeks. D. ETS-staining of the Rosenthal’s canal of the b2 half-turn of a deaf ear from Group 1 h, sacrificed after a follow-up of 26 weeks. A further reduction of nuclei can be observed. Scale bar: 50 µm. E. SGN densities of the different half-turns of ears with normal hearing and of the deaf ears of Group 1 h, sacrificed 5, 14, then 26 weeks after deafening. A significant reduction of SGN density can be seen in all half-turns already after 5 weeks. Furthermore, significantly lower densities in all cochlear half-turns could be observed after 14 weeks.
necessary. Complete deafness could be achieved in these ears.

Only one ear was treated with the deafening solution in 2 animals, which resulted in severe vestibular symptoms with spontaneous nystagmus. These animals needed intense postoperative care. The symptoms disappeared after 2–3 days.

4. Discussion

Deafening protocols for research purposes have already been applied for decades. For electrophysiological or morphological studies, acoustic trauma is preferred by many authors as hearing-impaired animal model (Ohlemiller, 2008; Rüttiger et al., 2013). However, the severity of hearing loss depends on many physical properties of the noise, such as intensity, exposure time, possible repetition rate, and interval length (Clark et al., 1987). In addition, the genetic background and the age of the animals may have an influence on the final result (Ohlemiller et al., 2011). The ototoxicity of chemotherapy drugs such as cisplatin has also been widely investigated (Rybak et al., 2007; Van Ruijven et al., 2005). The effect of different otoprotective agents on cisplatin-treated animals can be tested with these models (Dickey et al., 2005; Gündoğdu et al., 2019).

The deafening method for cochlear implant experiments should lead to stable and irreversible HC loss. Therefore, systemic application of kanamycin and furosemide is the most frequently used method. Unfortunately, nephrotoxicity can occur as a side effect, which can even be fatal. In addition, unreliable and reversible hearing losses have been reported using those methods (Aran et al., 1975; Shepherd et al., 2005). Shepherd et al. (2005) had to exclude animals before growth factor treatment because inadequate hearing loss was achieved. Aran et al. (1975) reported a reversible effect with kanamycin treatment. The intratympanic application of combination of kanamycin and furosemide has already been used in rats (Murillo-Cuesta et al., 2009). Investigating the ABR threshold shifts after deafening with systemic or local administration of the drugs, no significant difference was found 1 week after the application, however the ABR-click threshold shifts were significantly lower in the locally treated animals. In the present study, we used the same solution for our procedure like (Murillo-Cuesta et al., 2009) but the trial design, the drug application method and the species were different. Drug exposure occurred only over the defined periods of 1 and 2 h, respectively, and thus was more controlled in its effect than with soaked gel foam. Moreover, we performed a substantially longer audiological follow-up (with CAP measurements instead of ABR) until 26 weeks in contrast to 2 weeks with a more detailed histological analysis and performed CAP measurements, which is beneficial especially in relation to unilateral deafness.

In our study we developed a method that leads to complete deafness in most animals. With a combination of 200 mg/ml kanamycin and 50 mg/ml furosemide administered intratympanically for 2 h, no hearing could be detected in 94% of the ears, whereas deafness was achieved in only 80% of the ears with the 1-hour treatment. During regular hearing measurements for 26 weeks, we could not detect any hearing improvement; the effect can be regarded as stable and irreversible. Moreover, no adverse health effects except vestibular symptoms were observed even in the animals monitored for 26 weeks.

In addition to these advantages, our method can also be applied for a single-sided deafness animal model although challenges can be raised due to the unilateral application. Temporary vestibular symptoms can occur, which require intensive postoperative care of the animals. In addition, asymmetrical hearing is a particular challenge in hearing measurements in the case of small animals. Frequently used ABR measurements have the disadvantage that the electrical potentials coming from the two ears cannot be distinguished from each other. To address this problem, we used electrophysiology (CAP) as a hearing measurement, which also allowed us to precisely detect potential residual hearing.

Hair cell loss due to administration of aminoglycosides has been widely reported and studied (Odosdon, 1997; Ylikoski et al., 1974). It is believed that SGNs degenerate secondarily after HC loss, at least in animals. However, investigating the surviving SGNs is crucial because these are the target cells for cochlear implants (Odosdon and Mohuiddin, 2006).

Animals treated with our method suffered severe HC loss, which was almost complete in the basal cochlear turn. We also showed that SGN degeneration occurs, and a 2–3 times lower packing density was measured 5 weeks after the deafening procedure, which is consistent with the findings of (Versnel et al., 2007) after systemic application of the drug combination. A strong correlation between hearing loss and HC and SGN degeneration has also been demonstrated (Versnel et al., 2007).
2007). Notably, the pathophysiology of the deafening effect was not analyzed in our study. It is believed that drugs mainly reach the inner ear fluid space through the round window membrane by diffusion (Salt and Ma, 2001). However, (Mikulec et al., 2009) reported a mechanism through the bony cochlear wall using gentamicin. As shown in Fig. 5, a partially deafened animal showed more severe hearing loss and hair cell loss in the basal turns (at the high frequency range). Therefore, we can assume that the deafening solution we used affected the basal cochlear part most likely through round window diffusion.

It should also be stated that the experiment was intended to prove the long-term effect of this deafening procedure and the survival of the animal up till 26 weeks. We showed that SGN degeneration after 5 weeks the hair cells loss was already achieved but the SGN degeneration was still in progress, and the basal turn of the cochlea was more affected in this timepoint. However further, short-term investigation is needed to clarify the dynamics of the hair cell and spiral ganglion neuron loss.

5. Conclusions

We studied the effect of the intratympanic application of a solution of 200 mg/ml kanamycin and 50 mg/ml furosemide in guinea pigs. A reliable and safe deafening method resulting in pancochlear HC damage was demonstrated using our protocol. We recommended the exposure time of 2 h because the higher percentage of deaf ears was achieved without additional SGN degeneration compared to the 1-hour experiment. Due to local administration, this method is free from the systemic side effects of aminoglycosides and loop diuretics, and it can also be used for modeling single-sided deafness.

CRediT authorship contribution statement

Peter Bako: Investigation, Formal analysis, Writing – original draft.
Imre Gerlinger: Writing – review & editing.
Stephan Wolpert: Writing – review & editing.
Marcus Müller: Conceptualization, Methodology, Formal analysis.
Hubert Lowenheim: Funding acquisition, Supervision.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adelman, R.D., Spangler, W.L., Beasom, F., Ishizaki, G., Conzelman, G.M., 1979. Furosemide enhancement of experimental gentamicin nephrotoxicity: comparison of functional and morphological changes with activities of urinary enzymes. J. Infect. Dis. 140, 342–352. https://doi.org/10.1093/infdis/140.3.342.
Aran, J.M., Darrouzet, J., Erre, J.P., 1975. Observation of click-evoked compound viii nerve responses before, during, and over seven months after kanamycin treatment in the adult mouse cochlea. J. Neurosci. 5, 13–13. https://doi.org/10.1021/bi5171000.
McLean, W.J., Hinton, A.S., Herby, J.T., Salt, A.N., Hartsock, J.J., Wilson, S., Lucchino, D.L., Lenarz, Thomas Warnecke, Athanasia Prenzler, N., Schmitt, U., King, S., Jackson, Lance E. Rosenblom, Jeffrey Airey, G., Bear, M., Runde, Christina L., Giffoni, D., Rauch, S.D., Lee, D.J., Langer, R., Karp, J.M., Loore, C., Leliev, C., 2021. Improved Speech intelligibility in Subjects with Stabile Sensorineural hearing loss Following Intratympanic Dosing of FX-322 in a Phase 1b Study. Otol. Neuror. Mikulec, A.A., Plonle, S.K., Hartsock, J.J., Salt, A.N., 2009. Entry of substances into perilymph through the bone of the otic capsule after intratympanic applications in guinea pig: implications for local drug delivery in humans. Otol. Neurol. Salt, A.N., Ma, 2001. The role of perilymph flow in the regulation of otic capsule oxygen tension. Arch. Otolaryngol. 127, 154–156. https://doi.org/10.1001/archotol.127.3.154.
Müller, M., Von Hörnstein, K., Hoids, S., Smolders, J.W.T., 2005. A physiological place-frequency map of the cochlea in the CBA/J mouse. Hear. Res. 202, 63–73. https://doi.org/10.1016/j.heares.2004.08.011.
Muirul-Cuesta, S., Garcia-Alcantara, F., Vaccas, E., Sistiaga, J.A., Camarero, G., Varelo-Nieto, L., Rivera, T., 2009. Direct drug application to the round window: a comparative study of ototoxicity in rats. Otolaryngol. Head Neck Surg. 141, 584–590. https://doi.org/10.1016/j.otohns.2009.07.014.
Ohlemiller, K.K., 2008. Recent findings and emerging questions in cochlear noise injury. Hear. Res. 245, 5–17. https://doi.org/10.1016/j.heares.2008.08.007.
Ohlemiller, K.K., Rybak Rice, M.E., Rellinger, E.A., Ortmann, A.J., 2011. Divergence of noise vulnerability in cochlea of young CBA/J and CBA/Ca mice. Hear. Res. 272, 13–20. https://doi.org/10.1016/j.heares.2010.11.006.
Rodriguez Salguero, X., Gonzalez-Nunez, L., 2016. Animal models mimicking aminoglycoside-induced renal damage. J. Neuropharmacol. 5, 1–3.
Rüttiger, L., Singer, W., Panford-Walsh, R., Matsumoto, M., Lee, S.C., Zuccotti, A., Zimmermann, U., Jaumann, M., Robbok, K., Xiong, H., Knipper, M., 2013. The reduced cochlear output and the failure to adapt the central auditory response causes tinnitus in noise exposed rats. PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0052747.
Rybak, L.P., Whitworth, C.A., Makhjerda, D., Ramkumar, V., 2007. Mechanisms of cipistatin-induced ototoxicity and prevention. Hear. Res. 226, 157–167. https://doi.org/10.1016/j.heares.2007.03.003.
Salt, A.N., Ma, Y., 2001. Quantification of solute entry into cochlear perilymph through the round window membrane. Hear. Res. 154, 88–97. https://doi.org/10.1016/S0378-5955(01)00223-9.
Shepherd, R.K., Coorn, E., Epp, S.B., Crook, J.M., 2005. Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss. J. Comp. Neurol. 486, 145–158. https://doi.org/10.1002/cne.20564.
Smith, G.R., Lietman, P.S., 1983. Effect of furosemide on aminoglycoside-induced nephrotoxicity and auditory toxicity in humans. Anticancer Agents Chemother. 23, 133–137. https://doi.org/10.1128/AAC.23.1.133.
Song, B.B., Sha, S.H., Schacht, J., 1998. Iron chelators protect from aminoglycoside-induced cochlear- and vestibulo-toxicity. Free Radic. Biol. Med. 25, 189–195. https://doi.org/10.1016/S0891-5849(98)00037-9.
Van Ruijven, M.W.M., De Groot, J.C.M.J., Klis, S.F.L., Smoorenburg, G.F., 2005. The cochlear targets of cisplatin: an electrophysiological and morphological time-course study. Hear. Res. 205, 241–248. https://doi.org/10.1016/j.heares.2005.03.023.
Vesnuel, H., Agerter, M.J.H., De Groot, J.C.M.J., Klis, S.F.L., Smoorenburg, G.F., 2007. Time course of cochlear electrophysiology and morphology after combined administration of kanamycin and furosemide. Hear. Res. 231, 1–12. https://doi.org/10.1016/j.heares.2007.03.003.
West, B.A., Brummett, R.E., Himes, D.L., 1973. Interaction of kanamycin and ethacrynic acid ethacrynic. Arch. Otolaryngol. 98, 32–37.

Wilson, B.S., Tucci, D.L., O’Donoghue, G.M., Merson, M.H., Frankish, H., 2019. A lancet commission to address the global burden of hearing loss. Lancet 393, 2106–2108. https://doi.org/10.1016/S0140-6736(19)30484-2.

Ylikoski, J., Wersäll, J., Björkroth, B., 1974. Degeneration of neural elements in the cochlea of the Guinea-pig after damage to the organ of corti by ototoxic antibiotics. Acta Otolaryngol. 78, 23–41. https://doi.org/10.3109/00016487409129730.