厚生労働科学研究研究費補助金

障害者対策総合研究事業（感覚器障害分野）

新世代人工内耳に対応した内耳薬剤徐放技術の開発

平成22年度　総括・分担研究報告書

研究代表者　吉　川　弥　生

平成23（2011）年3月

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統括研究報告書

新世代人工内耳に対応した内耳薬剤徐放技術の開発

研究代表者　吉川　弥生　東京大学医学部附属病院　耳鼻咽喉科・頭頸部外科
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研究要旨

人工内耳治療には、近年になり適応拡大・両耳装用、さらには海外での人工内耳と補聴器の併用（EAS）といった大きなパラダイム・シフトが起きている。これを実現するには、埋込術後の急性期の蝸牛障害を予防する技術の開発が必須である。

本研究課題では、研究代表者らが有するバイオマテリアル技術および内耳アポトーシス予防技術を統合し、薬剤徐放機能付き人工内耳などの新たな内耳治療手技の開発を行った。

ゼラチン・ハイドロゲルを用いたダミー人工内耳電極を作成したところ、IGF-1 および HGF-ハイドロゲル製剤は、人工内耳挿入時の蝸牛障害を有意に軽減する効果を持つことが明らかになった。今後、徐放性能の最適化および臨床試験への準備を進めて行く。

分担研究者
坂本　幸士　東京大学医学部附属病院　耳鼻咽喉科・頭頸部外科　講師
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松本　有　東京大学医学系研究科　臨床医工学部門　特任研究員
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A．研究目的

新世代人工内耳の発展

人工内耳は 20 世紀最高の発明とも言われ、最も成功した人工臓器のひとつである。1970 年代の実用化以降、単チャンネルからマルチチャンネルへ、スピーチプロセッサの IC 化といった様々な改良がなされてきたが、近年になり大きなパラダイム・シフトが起きている。それは、人工内耳自体の性能向上に沿った中等度難聴への適応拡大・両耳装用、さらには人工内耳と補聴器の併用（EAS）などに代表される「人工内耳治療の普遍化」である（Van de Heyning 2010）。EAS は欧州ではすでに 10 年前より臨床での使用が始まっているが、日本でも本年 12 月 9 日に厚生労働省「高度医療評価会議」で承認され、普及への第 1 歩を踏み出した。

しかしながら、こうした新世代人工内耳に対応した内耳保護技術は必ずしも充分ではない。EAS を行うためには残存聴力の温存が必須であるが、電極挿入により起こる内耳組織破壊・繊維化などのために不可逆的に喪失してしまうことが多い（Nadol 1997 など）。したがってより安全で低侵襲

な人工内耳手術術式を開発するとともに、手術時には繊維化を防ぎ、蝸牛細胞を保護・再生する薬剤を内耳局所に投与することが望まれている。

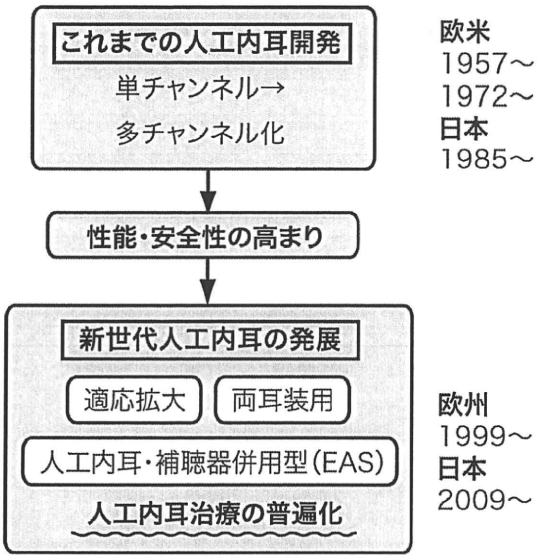


図１　人工内耳の発展

人工内耳を利用した薬剤投与技術の開発が急務である

人工内耳からの薬剤投与に関しては現在世界的に熾烈な競争が繰り広げられているが、浸透圧ポンプなどを使うと装置が巨大化してしまうことや、効果の高い薬剤が入手できないといった理由からいずれも学会報告レベルに留まっている。

研究代表者はこれまで所属していたテキサス大学、京都大学で人工内耳の感染予防加工や低侵襲手術法の開発を行い（Med-El 社との共同研究）、内耳薬剤徐放の種々の技法を習得した。IGF-1・ハイドロゲル徐放製剤は動物実験で高い効果が得られ、突発性難聴に対する臨床第 I/II 相試験では５割に効果を認めた（BMC Medicine 2010, 8:76）。また、東京大学ではアポトーシス予防に関して基礎研究(Someya 2009 PNAS)、全身投与（Kashio 2007, J Neurosci Res）を通して技術を確立しており、本研究ではこの両者の技術を統合して内耳薬剤徐放機能を備えた低侵襲型人工内耳を開発、人工内耳埋込時に起きる組織損傷を極限まで抑える技術を開発する。

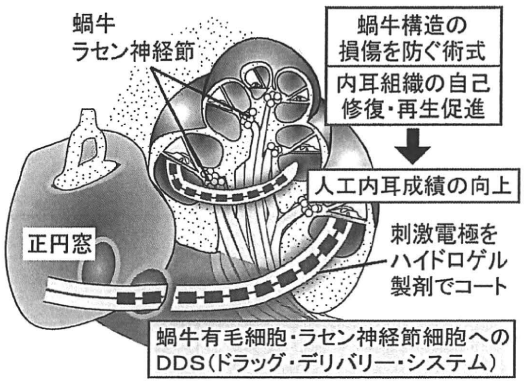


図２　本計画の概念図

さらにこの技術が完成すれば、人工内耳治療に限らず突発性難聴や進行性難聴などの様々な内耳疾患の普遍的な治療法として応用が期待できると考えられた。

B．研究方法

１）人工内耳電極コート技術の開発

ハイドロゲルコート電極のプロトタイプを作成し、*in vitro* 徐放試験やIGF-1、HGFを使った動物実験により薬剤徐放機能・人工内耳挿入時損傷防止効果を測定する。シリコン電極の親水化コート方式としてはプラズマ放電を用いるが、通電試験（5-30mA）により電極性能の低下やゲル剥脱などの問題が認められた場合にはＵＶ照射あるいは O_3（オゾン）処理を検討する。

・ゼラチン担体からの *in vitro* 薬物徐放試験

1．コート済みダミー電極をサンプリングチューブに分け入れ、I^{123} ラベル IGF-1 溶液 1ml を滴下して電極と触れる状態にし、室温 3 時間（もしくは 37℃ 1 時間）静置して含浸させる。（n=3 程度）

2．PBS を 1ml ずつ加え、37℃恒温槽で浸とうしながら薬物を拡散放出させる。

3．0.5, 1, 2, 4, 8, 12, 24 hr 後に、PBS を全量抜き取り、サンプル溶液とする。PBS 1ml を新たに加え、引き続き 37℃で浸とうする。

4．それぞれの時間に採取したサンプル溶液中の薬物濃度を算出し、累積して放出量を計算する。全てサンプリング後、可能であれば残

存量を測定して合計量の確認を行う。

2）齧歯類モデルでの動物実験

開発した人工内耳電極を内耳に挿入して、実際の薬物徐放を行い、薬物の生物学的有効性を齧歯類モデルで解析する。内耳機能の解析方法としては、聴力検査（ABR)、神経反応テレメトリ（NRT)、凍結切片を用いた組織学的検査を行う。

・モルモット蝸牛損傷予防実験

1．コート済みダミー電極をサンプリングチューブに分け入れ、IGF-1 または HGF 溶液 0.5ml を滴下して電極と触れる状態にし、4℃で一昼夜静置して含浸させる。（n=5 程度）

2．モルモット側頭部に耳後切開を置いて耳胞を開放、蝸牛開窓を置きそこから薬剤含浸ダミー電極を挿入する。

3．術前、術直後、３、７、14、21、28 日後に ABR を測定する。

4．28 日後に蝸牛を回収し中耳および内耳の組織学的検査を行う。

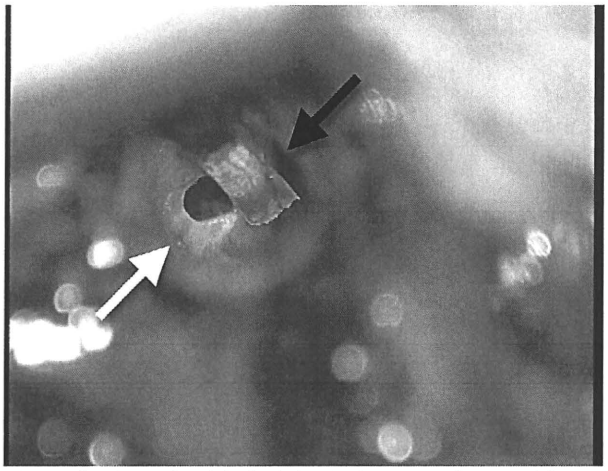


図　蝸牛開窓（白矢印）を行い、ダミー電極（黒矢印）を挿入

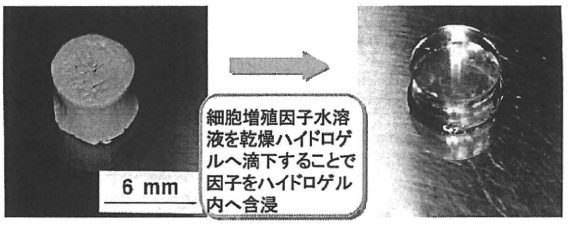
（倫理面への配慮）

動物実験に関しては、本学の動物実験に関する倫理委員会の承認のもとに、動物愛護に十分配慮した上で行う。ヒト側頭骨を使用する場合は、人権擁護上の配慮を十分に行った上で研究を実施する。

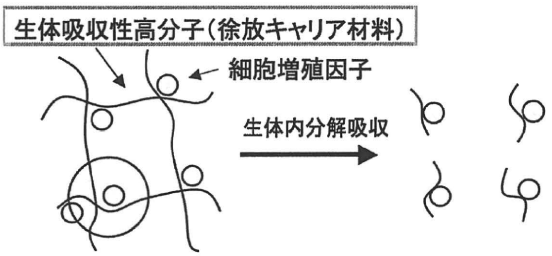
C．研究結果

1）人工内耳電極コート技術の開発

ゼラチン・ハイドロゲルを用い、コート電極のプロトタイプを作成した。プラズマ放電を用いてシリコーン表面を親水化することで、ハイドロゲル膜の形成が可能であった。

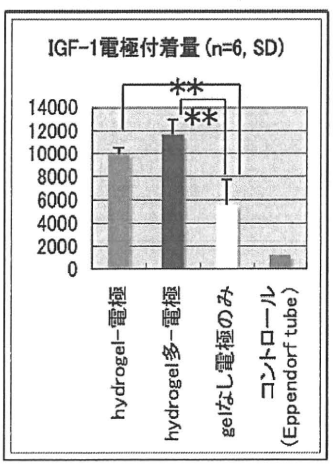


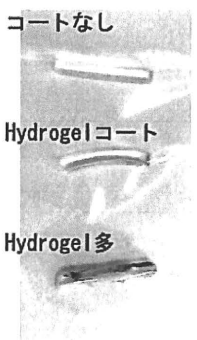
凍結乾燥ハイドロゲル　　細胞増殖因子を含浸したハイドロゲル



・ *in vitro*薬剤電極吸着試験

I^{123}ラベルIGF-1を用いて測定したところ、ハイドロゲル付着ダミー電極は、ゲル層を持たない電極に比べて有意にIGF-1を多く吸着していた。





（ニンヒドリンで呈色）

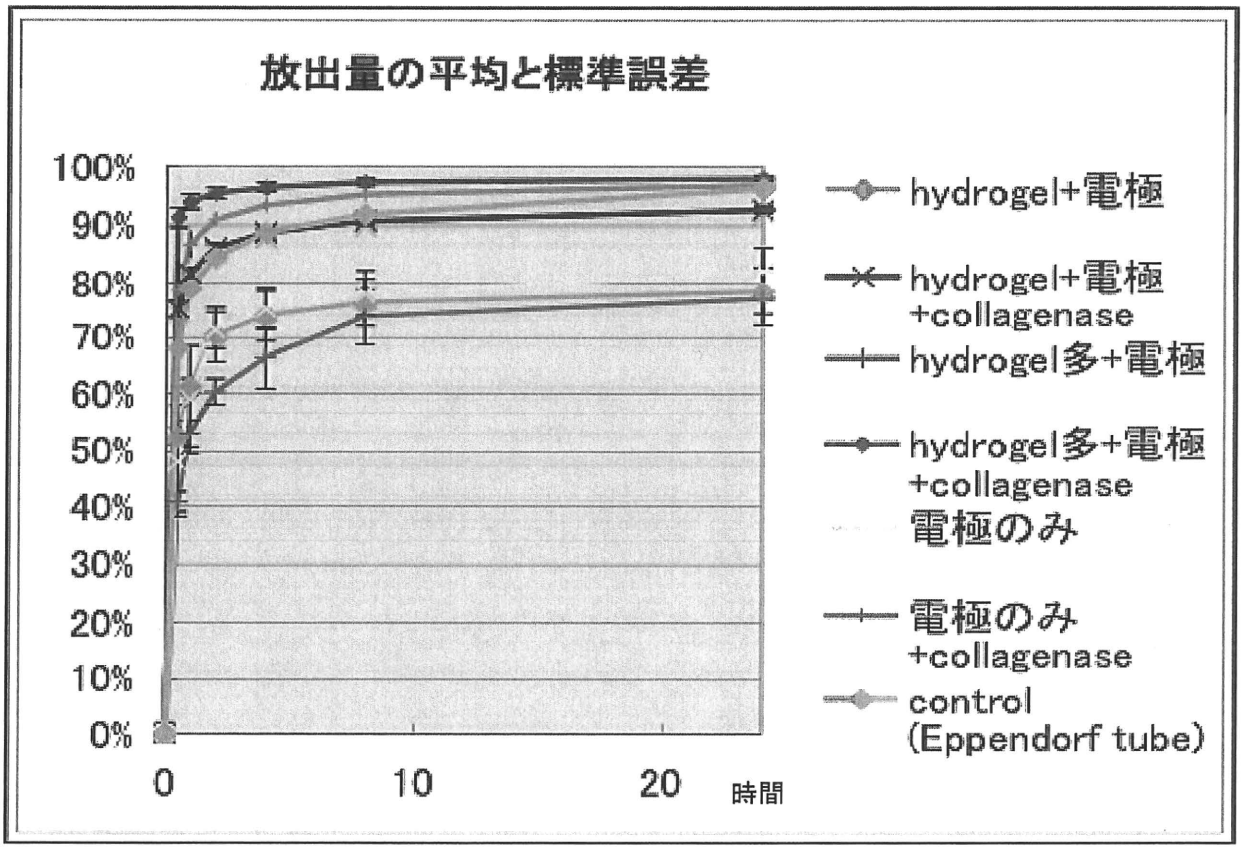


図　*in vitro* 薬剤放出試験

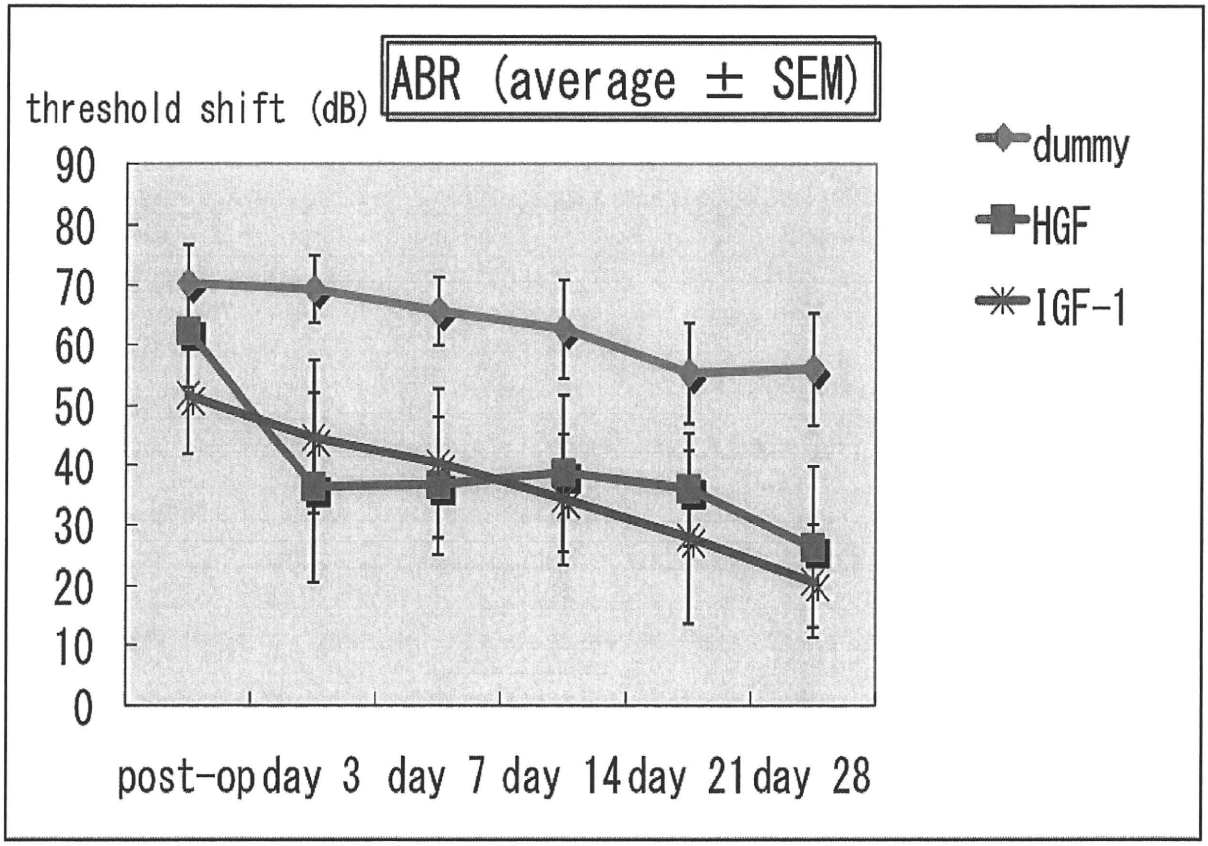


図　モルモット蝸牛開窓実験

・ *in vitro*薬剤徐放試験

I^{123}ラベルIGF-1を用いて測定したところ、ハイドロゲル付着ダミー電極は、ゲル量の多い場合にIGF-1保持量が最も多かった。また、ゲル量が多くさらに溶液中にコラゲナーゼを添加した場合に最も早くコラゲナーゼを放出した。

2）齧歯類モデルでの動物実験

モルモットを使った電極挿入実験では（未公開データ）、HGF含浸電極、IGF-1含浸電極ともに無薬剤電極に比べて有意に電極挿入時以降のABRの回復が早かった。（ダミー電極 vs HGF/ハイドロゲル: $P=0.00018$、ダミー電極 vs IGF-1/ハイドロゲル: $P=0.0000065$）

組織学的解析では、内耳及び中耳に特に強い炎症反応は認められなかった。

なお、薬剤を含んだゼラチン・ハイドロゲル層が電極挿入時に電極から剥奪する現象が高い割合で発生した。

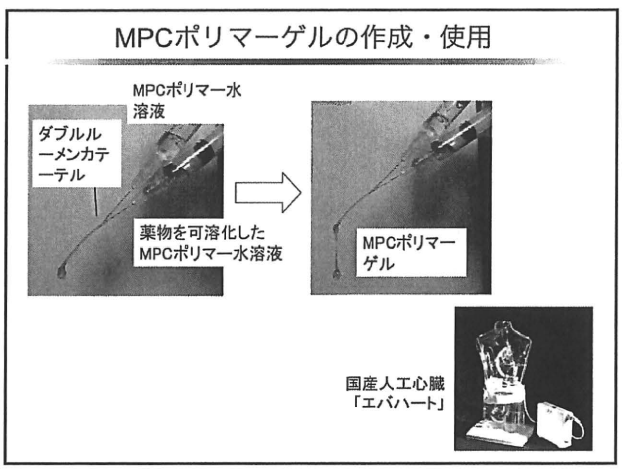
D．考察

1）徐放製剤を用いた蝸牛への薬物投与

本研究では、ゼラチン・ハイドロゲルで被覆した人工内耳ダミー電極について、内耳用徐放製剤として使用でき、ゲル量に応じて薬剤徐放速度と徐放時間を制御できることが確認できた。

しかしながら、動物実験でダミー電極を挿入した際にハイドロゲル層が剥奪する事故が発生しうることが判明した。この問題を回避するには、1）UV照射あるいはO_3（オゾン）処理など、現在のプラズマ放電に代わるシリコーン表面処理方法を検討する　2）ハイドロゲル以外の徐放材料を検討する　の二つの方法が考えられる。

2）に関しては、現在検討中の徐放材料として①ナノミセル型DDS（Nishiyama 2003、東大医工連携　片岡一則教授）　②Tetra-PEG gel（Kurakazu 2010、東大工学部（マテリアル工学専攻）酒井崇匡助教）、③MPCポリマーゲル（Kihara 2003、東大工学部（先端バイオデバイス工学）石原教授、金野准教授）、の３つがある。①はダハプラチン内包ナノミセル抗がん剤としてPhase III試験中、②は骨再生材料として、③は国産で初めて承認された埋込型補助人工心臓「エバハート」の血栓予防のための表面コーティングとして、それぞれ臨床応用されている。MPCポリマーは、２剤の配合割合により脂溶性の薬剤についても可溶化・ゲル化することができるため特に有望と考えられる。

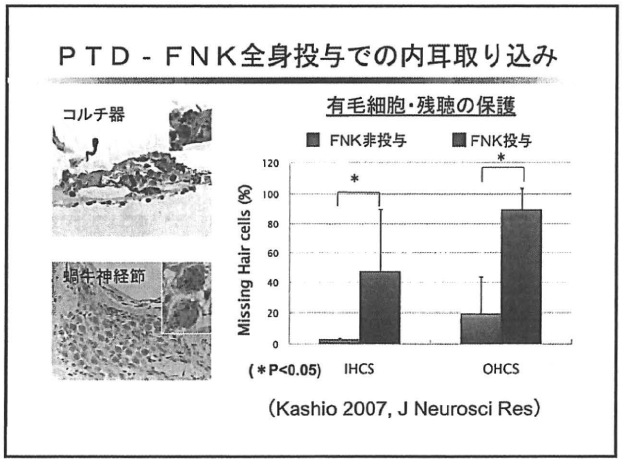


2）徐放薬剤の選定

本研究ではIGF-1およびHGFを徐放薬剤として使用したが、蝸牛障害抑制効果はIGF-1に強く認められたものの、HGF-1との有意差はなかった。このうちIGF-1（インスリン様細胞成長因子）・ハイドロゲル徐放製剤の内耳局所投与に関しては突発性難聴に対する世界初の内耳DDS臨床第Ⅰ/Ⅱ相試験を行い、有意な治療効果を確認している。

今後は、ステロイドなどの脂溶性薬剤、あるいはPTD-FNKなどの別の機序を有する薬剤についても検討を行う。

PTD-FNK蛋白とは、アポトーシス抑制FNK蛋白に、HIV遺伝子産物のTat蛋白の一部であるPTDを付加したものである。これを使用した内耳投与実験（ゼラチンスポンジを使用）では、騒音難聴動物に対する蝸牛局所投与で、一定の障害抑制効果を確認している（Kashio 2007, J Neurosci Res）。



3）今後の研究について

今後、本研究をさらに進めて行くにあたり、以下の研究を予定している。

平成23年４月～　電極通電試験
動物実験
低侵襲手術法開発

平成23年10月～　特許申請および知財化

E．結論

本研究課題では、研究代表者らが有するバイオマテリアル技術および内耳アポトーシス予防技術を統合し、薬剤徐放機能付き人工内耳などの新たな内耳治療手技の開発を行った。IGF-1およびHGF-ハイドロゲル製剤は、人工内耳挿入時の蝸牛障害を有意に軽減する効果を持つことが明らかになった。今後、徐放性能の最適化および臨床試験への準備を進めて行く。

F．健康危険情報

現時点では、ヒトにおける健康危険に関する情報は得られていない。

G．研究発表

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研究成果の刊行に関する一覧表

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Topical insulin-like growth factor 1 treatment using gelatin hydrogels for glucocorticoid-resistant sudden sensorineural hearing loss: a prospective clinical trial

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Abstract

Background: Sudden sensorineural hearing loss (SSHL) is a common condition in which patients lose the hearing in one ear within 3 days. Systemic glucocorticoid treatments have been used as standard therapy for SSHL; however, about 20% of patients do not respond. We tested the safety and efficacy of topical insulin-like growth factor 1 (IGF1) application using gelatin hydrogels as a treatment for SSHL.

Methods: Patients with SSHL that showed no recovery to systemic glucocorticoid administration were recruited. We applied gelatin hydrogels, impregnated with recombinant human IGF1, into the middle ear. The primary outcome measure was the proportion of patients showing hearing improvement 12 weeks after the test treatment. The secondary outcome measures were the proportion of patients showing improvement at 24 weeks and the incidence of adverse events. The null hypothesis was that 33% of patients would show hearing improvement, as was reported for a historical control after hyperbaric oxygen therapy.

Results: In total, 25 patients received the test treatment at a median of 23 days (range 15-32) after the onset of SSHL, between 2007 and 2009. At 12 weeks after the test treatment, 48% (95% CI 28% to 69%; $P = 0.086$) of patients showed hearing improvement, and the proportion increased to 56% (95% CI 35% to 76%; $P = 0.015$) at 24 weeks. No serious adverse events were observed.

Conclusions: Topical IGF1 application using gelatin hydrogels is well tolerated and may be efficacious for hearing recovery in patients with SSHL that is resistant to systemic glucocorticoids.

Background

Sudden sensorineural hearing loss (SSHL) is a condition in which an individual experiences hearing loss of at least 30 dB over at least three test frequencies in one ear within a period of 3 days [1]. Some patients recover completely without medical intervention, often within the first 3 days. Others get better slowly over a 1-week or 2-week period, which is known as 'spontaneous recovery' [1]. Although a good recovery is likely, 15% of patients with SSHL experience hearing loss that worsens over time. Approximately 40,000 new cases of SSHL occur each year in the US [1], and 35,000 patients with SSHL consult a doctor each year in Japan [2]. SSHL can affect anyone; however, for reasons that so far remain unknown, it is most often reported in people aged between 30 and 60 years. The most common therapy for SSHL is the systemic application of glucocorticoids. Unfortunately, about 20% of patients do not respond to this treatment [3].

Based on these findings, researchers have sought alternative therapeutic options for SSHL. Protecting auditory hair cells and primary neurons from irreversible

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degeneration is a practical strategy, as inner ear cells have limited regeneration capacity [4]. Recent improvements in our understanding of the role of growth factors in the maintenance of mature peripheral auditory systems have led to numerous attempts to define ways to reduce auditory hair cell and neuron degeneration, which have indicated that some growth factors have potential for the treatment of SSHL [5-8]. However, growth factors have not yet been used for this purpose in a clinical setting, as several obstacles have hindered their progress. Safe and effective methods for the sustained delivery of growth factors to the inner ear need to be developed to facilitate their clinical application. As a solution to this problem, we used gelatin hydrogels as a vehicle to deliver growth factors to the inner ear [9]. Gelatin hydrogels consist of gelatin polymers that are electrostatically complexed with growth factors [10]. The growth factors are released by the enzymatic degradation of the gelatin polymers after application. Our focus was on insulin-like growth factor 1 (IGF1), which has been approved for clinical application. We conducted a series of animal experiments, which revealed that topical IGF1 application via gelatin hydrogels significantly improved hearing by protecting auditory hair cells against damage caused by intense noise exposure [11] or ischaemic injury [12]. Moreover, no adverse events were observed in animals following the local application of IGF1 via gelatin hydrogels [11].

Here, we report on a prospective clinical trial of topical IGF1 application through gelatin hydrogels for the treatment of glucocorticoid-resistant SSHL, which was intended to provide preliminary estimates of variables for generating hypotheses for more specific studies using randomised trials when appropriate. Systemic glucocorticoid application has been regarded as a primary treatment of choice for SSHL. We recruited patients with SSHL that showed no recovery to systemic glucocorticoid administration as subjects in the present study.

Methods

Patients

Patients were eligible for inclusion in the study if they met the following conditions: they had been diagnosed between December 2007 and July 2009 at the Department of Otolaryngology, Head and Neck Surgery of Kyoto University Hospital, Japan as having definite or probable SSHL within 29 days of onset; they presented with an abnormality in evoked otoacoustic emission, which indicated dysfunction of the auditory hair cells; no recovery was determined according to the criteria for hearing improvement as set by the Sudden Deafness Research Committee of the Japanese Ministry of Health, Labour and Welfare in 1984 [13] (Table 1) more than 7 days after systemic glucocorticoid treatment; and they were aged over 20 years. We excluded patients with active chronic otitis media, acute otitis media, otitis media with effusion or dysfunction of the auditory tube, a history of previous treatments (except for systemic application of glucocorticoids or prostaglandin E1), malignant tumours, severe liver dysfunction (aspartate aminotransferase (AST) >100 IU/L and alanine aminotransferase (ALT) >100 IU/L), uncontrolled diabetes (haemoglobin A1C (HbA1c) >10%), pituitary or adrenal dysfunction, severe systemic illness that affected life expectancy, a history of severe drug allergy, or a history of alcohol or drug dependence within the past 1 year, and pregnant or lactating women. Magnetic resonance imaging (MRI) was performed on all patients to rule out acoustic neurinoma.

This study was single arm, non-randomised and open. Placebo applications and blinding were not used, as it was anticipated that they would have reduced compliance.

The primary outcome measure was the proportion of patients showing hearing improvement, which was defined as better than slight recovery according to the criteria shown in Table 1, 12 weeks after the test treatment. The secondary outcome measures were the proportion of patients showing hearing improvement 24 weeks after the test treatment and the incidence of adverse events during the observation period.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and its amendments, and approved by the Ethical Committee of the Graduate School of Medicine, Kyoto University (registered number, C165). Each patient gave written, informed consent to participate in this study.

Trial registration

This trial was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) on 6 December 2007 under trial registration number UMIN-CTRR000000936.

Procedures

The test treatment was performed within 4 days of registration. Gelatin hydrogels were made from porcine skin gelatin (Nitta Gelatin Inc., Osaka, Japan) in a clean room at the Department of Pharmacy, Kyoto University Hospital, according to the method described previously [14], and were preserved at temperatures below 4°C before use. Procedures for topical IGF1 treatment were performed in the Day-Surgery Unit of Kyoto University Hospital. Mecasermin (recombinant human IGF1 (Somazon), 10 mg injection; Astellas Pharma Inc., Tokyo, Japan) was dissolved in physiological saline at a final concentration of 10 mg/ml. A 30 μl sample of

Table 1 Criteria for hearing improvement determined by the Sudden Deafness Research Committee of the Japanese Ministry of Health, Labour and Welfare in 1984

Improvement	Criteria
Complete recovery	Recovery of a hearing level within 20 dB at all five frequencies tested (0.25, 0.5, 1.0, 2.0 and 4.0 kHz) or recovery to the same level as the opposite side in pure tone auditometry
Marked recovery	More than 30 dB recovery in the mean hearing level at the five frequencies tested
Slight recovery	Recovery of 10 to 29 dB in the mean hearing level at the five frequencies tested
No recovery	Less recovery than 10 dB in the mean hearing level at the five frequencies tested

mecasermin solution was mixed with 3 mg of gelatin hydrogels 60 min before application. The hydrogel containing 300 μg of mecasermin was placed in the round-window niche of the middle ear following tympanostomy under local anaesthesia with 1% lidocaine. A single application was used. Patients were hospitalised for 4 days after the surgical procedure, and their general and local conditions were examined at the outpatient clinic of the Department of Otolaryngology, Head and Neck Surgery, Kyoto University Hospital, for 24 weeks after the test treatment. Pure-tone audiometry and evoked otoacoustic emission were measured on the day of registration, at 3 days after the test treatment, and at 1, 2, 4, 12 and 24 weeks after the test treatment. During the observation period, all adverse events were recorded.

Statistical analysis

The threshold improvement (33%, 66/199) was based on a historical control of hyperbaric oxygen therapy (19 times in total; range 5-55) for 199 patients with glucocorticoid-resistant SSHL at Kyoto University Hospital between October 2000 and September 2006 [15]. The null hypothesis was that the proportion of patients with hearing improvement at 12 or 24 weeks after the test treatment would be equivalent to the proportion of patients with hearing improvement reported in a historical control administered hyperbaric oxygen therapy. The sample size was based on binominal distribution with a one-sided significance level of 0.05 and a power of 0.90 (expected proportion of 63%). The required sample size was 25 after considering 10% (3 samples) of patients who would be excluded from the analysis. The null hypothesis was rejected at the 0.05 level of probability (one-sided) based on a binominal distribution. Statistical analyses were performed using SAS v.9.2 (SAS Institute Inc. Cary, NC, USA).

Results

In all, 26 patients fulfilled the inclusion criteria, 1 of whom was excluded before the test treatment because of a diagnosis of functional hearing loss. In total, 25 patients (13 women and 12 men) were treated in accordance with the study protocol, and data for assessment of the primary and secondary outcomes were available for all patients. The median age at registration was 49 years (range 23-72 years). Comorbidities were found in 22 of the 25 patients (88%), and 18 of the 25 patients (72%) had a history of previous diseases. None of the comorbidities or previous diseases presented were directly associated with SSHL. None of the patients had family histories of SSHL. All 25 patients complained of associated symptoms: 22 (88%) complained of tinnitus, 19 (76%) had a feeling of ear fullness and 14 (56%) complained of dizziness. The median interval between the onset of SSHL and the initiation of the test treatment was 23 days (range 15-32 days). The mean hearing level at registration was 81.2 dB (95% confidence interval (CI), 71.2 to 91.1).

A summary of the hearing recovery according to pure-tone audiometry for all of the patients is shown in Table 2. At 12 weeks after the test treatment, 48% (95% CI 28% to 69%; $P = 0.086$) of the patients showed hearing improvement. The null hypothesis for the primary outcome was not rejected. Of the 25 patients, 0 showed complete recovery, 1 (4%) showed marked recovery, 11 (44%) showed slight recovery and 13 (52%) showed no recovery at 12 weeks. None of the patients who were treated more than 26 days after the onset of SSHL showed hearing improvement. At 24 weeks after the test treatment, the proportion of patients showing hearing improvement was 56% (95% CI 35% to 76%; $P = 0.015$), showing that the null hypothesis was rejected for the data at 24 weeks. Of the 25 patients, none showed complete recovery, 1 (4%) showed marked recovery, 13 (52%) showed slight recovery, and 11 (44%) showed no recovery. Two patients showed a hearing improvement of less than 10 dB at 12 weeks after the treatment, but an improvement of 10 dB at 24 weeks.

No serious adverse events associated with the test treatment occurred, although any adverse events were recorded in all of 25 patients to be evaluated. Adverse events with an incidence rate of more than 20% included dizziness (44%), nausea (24%), otitis externa (32%), common cold (20%) and otitis media (28%). All adverse events disappeared within the observation period. Except for two patients, the dizziness appeared either on the day of local IGF1 application or on the next day, and continued for a mean of 6.4 days (range

Table 2 Hearing recovery according to pure-tone audiometry

				Averaged hearing level (dB)			Hearing improvement	
Patient	Age	Gender	Days from onset	Before registration	12 weeks	24 weeks	12 weeks	24 weeks
1	54	M	19	88	77	75	SR	SR
2	36	F	31	62	55	60	NR	NR
3	46	M	21	107	81	86	SR	SR
4	29	F	24	107	95	95	SR	SR
5	38	M	19	65	64	62	NR	NR
6	72	M	29	98	97	97	NR	NR
7	49	M	17	111	105	105	NR	NR
8	49	F	26	47	42	42	NR	NR
9	55	M	21	104	78	75	SR	SR
10	55	F	29	52	57	57	NR	NR
11	60	F	27	37	33	32	NR	NR
12	35	F	21	76	68	66	NR	SR
13	59	M	23	90	79	78	SR	SR
14	58	M	32	60	81	77	NR	NR
15	60	F	26	63	40	39	SR	SR
16	36	M	19	56	51	46	NR	SR
17	33	F	18	88	88	87	NR	NR
18	61	F	25	92	72	74	SR	SR
19	42	F	15	111	89	92	SR	SR
20	23	F	18	79	22	18	MR	MR
21	45	F	26	95	82	77	SR	SR
22	45	M	28	87	84	85	NR	NR
23	60	F	23	108	84	86	SR	SR
24	26	M	20	109	92	86	SR	SR
25	55	M	21	37	34	35	NR	NR

Average hearing level was the mean hearing level according to pure-tone audiometry at the five frequencies tested (0.25, 0.5, 1.0, 2.0 and 4.0 kHz). Hearing improvement was determined by the criteria shown in Table 1.

MR = marked recovery; NR = no recovery; SR = slight recovery.

1-20 days). In all patients, the dizziness appeared after the test treatment. In one patient, dizziness appeared 2 months after the test treatment and continued for 4 months. In another patient, dizziness appeared 7 days after the application and disappeared 2 days later. Otitis media was found in 7 of the 25 (28%) patients, and was cured within a mean of 9.4 days (range 2-17 days). Exacerbation of tinnitus appeared in two patients at 29 and 33 days after the test treatment, respectively. None of the patients showed residual perforation of the tympanic membrane or additional hearing loss over 10 dB.

Discussion

Hearing loss is common, affecting about 5% to 6% of the population of the USA [1]. SSHL is one of the most common clinical conditions encountered by otolaryngologists, although it is less common than age-related hearing loss. National surveys have demonstrated the incidence of SSHL to be 5-30 per 100,000 per year [2,16,17]. Systemic application of glucocorticoids has been used as a standard therapy, although the supporting evidence is weak. Although systemic glucocorticoid application results in hearing recovery in some patients with SSHL, approximately 20% show no recovery [3]. Alternative therapeutic treatment options for SSHL have thus been eagerly sought. Against this background, we began developing topical IGF1 treatments using gelatin hydrogels in animal models [5,11,12], followed by a clinical trial to investigate their safety and efficacy for use in patients with SSHL. Some studies have indicated that SSHL develops when the inner ear does not receive a sufficient oxygen supply [18]. Consequently, hyperbaric oxygen treatment has been used as an alternative option for the treatment of SSHL [19,20]. At Kyoto University Hospital, hyperbaric oxygen therapy has been used as a secondary treatment of choice for glucocorticoid-resistant SSHL [14]. We thus used the proportion of patients with glucocorticoid-resistant SSHL showing hearing

recovery following hyperbaric oxygen therapy as a historical control.

Here, we report hearing recovery according to pure-tone audiometry and incidence of adverse events following topical IGF1 application using gelatin hydrogels in patients with SSHL enrolled in a single arm, non-randomised and open trial. Topical IGF1 treatment resulted in hearing recovery in approximately half of the patients with SSHL that had not responded to systemic glucocorticoid application, although the null hypothesis was rejected at 24 weeks after the test treatment but not at 12 weeks. In addition, no serious adverse events were observed during the 24-week observation period. The results indicated that the topical IGF1 application using gelatin hydrogels was safe, and had equivalent or superior efficiency to the hyperbaric oxygen therapy that was used as a historical control; this suggests that the efficacy of topical IGF1 application using gelatin hydrogels for SSHL that is resistant to systemic glucocorticoid treatments should be evaluated using randomised clinical trials.

Spontaneous recovery occurs in 40% to 65% of patients with SSHL [21,22], which makes it difficult to examine the exact therapeutic effects of interventions. It is therefore important either to eliminate patients with spontaneous recovery from such trials or to include a placebo control. In the present study, the test treatment was initiated in all patients more than 14 days (mean 23 days; range 15-32 days) after the onset of SSHL. In most cases, spontaneous recovery occurs within 14 days of onset [21]. We therefore consider spontaneous recovery to have had a negligible influence on the present results.

As a secondary treatment of choice for SSHL, intratympanic injection of glucocorticoids has gained considerable attention, because it seems to deliver a high concentration of glucocorticoids to the inner ear [23]. In addition, local application can reduce the total amount of glucocorticoids that needs to be applied, leading to a reduced risk of adverse events [24]. However, this approach remains controversial, because the criteria used to judge its efficacy differ in the literature. Haynes *et al.* [25] reviewed the literature on the intratympanic injection of glucocorticoids for SSHL after the failure of systemic treatment, and re-estimated the hearing recovery based on their own criteria, according to which a 20 dB improvement as indicated by pure-tone audiometry or a 20% improvement in discrimination was considered to be a successful therapeutic intervention. The recovery rates according to their criteria were 0% to 40%. When these criteria for successful intervention were applied to the data from the present study, the recovery rate was 24%, suggesting that the efficacy of topical IGF1 treatment using gelatin hydrogels might be equivalent to that of the intratympanic injection of glucocorticoids. We therefore recommend that the efficacy of topical IGF1 treatment using gelatin hydrogels should be evaluated in a randomised clinical trial, and its effectiveness for SSHL should be compared with that of the intratympanic injection of glucocorticoids.

Conclusions

The present results indicate the safety and efficacy of the use of topical IGF1 treatment using gelatin hydrogels for SSHL resistant to systemic glucocorticoid treatments. A double-blinded, randomised clinical study could clarify these findings. However, there are ethical obstacles to the use of double-blinded, randomised clinical trials for SSHL. For instance, the time from the onset of SSHL to the start of treatment has been regarded as important for the outcome, with prompt treatment preventing the development of irreversible auditory pathological changes. In addition, systemic glucocorticoid treatments have widely been accepted as a standard therapy for SSHL, and have led to improvement in some patients [26]. Hence, there would be ethical difficulties in not offering patients treatment with systemic glucocorticoids. Moreover, topical IGF1 application using gelatin hydrogels requires the use of surgical procedures, which would make it difficult to test in a double-blinded study. Therefore, as a next step, we will conduct a randomised clinical trial to compare the efficacy of topical IGF1 treatment using gelatin hydrogels with that of the intratympanic injection of glucocorticoids in patients with SSHL that is resistant to systemic glucocorticoids; it is hoped that this might clarify the efficacy of topical IGF1 treatment using gelatin hydrogels.

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Authors' contributions

TN, RO, SaT and JI planned the study. TS, HH, YSK and NM performed surgical treatment and collected the data. KH, KO, AY, KI, MY and YT prepared the gelatin hydrogels. SaT, ShT and HT analysed the data. TN wrote the article. JI edited the article.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Hydrogen protects vestibular hair cells from free radicals

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Abstract

Conclusion: Hydrogen gas effectively protected against the morphological and functional vestibular hair cell damage by reactive oxygen species (ROS). *Objective:* ROS are generally produced by oxidative stress. In the inner ear, ROS levels increase as a result of noise trauma and ototoxic drugs and induce damage. It is thus important to control ROS levels in the inner ear. The protective effects of hydrogen gas in cochlear hair cells have been reported previously. *Methods:* This study examined the effects of hydrogen gas on mouse vestibular hair cell damage by ROS using antimycin A. *Results:* In the group *exposed to hydrogen gas, vestibular hair cells were morphologically well preserved and their mechano-electrical transduction activities were relatively well maintained when compared with controls. Hydroxyphenyl fluorescein (HPF) fluorescence in vestibular tissue was also reduced by hydrogen gas.

Keywords: *Reactive oxygen species, vestibular hair cells, mechano-electrical transduction*

Introduction

Numerous patients suffer from balance disorders and the number increases with age. Vestibular hair cells also degenerate with age [1] and are damaged by oxidative stress [2]. Oxidative stress is one of the most important factors related to disorders of the inner ear [3–5]. In the inner ear, levels of reactive oxygen species (ROS) increase as a result of noise trauma and ototoxic drugs [6–10] and induce damage. It is thus important to control ROS levels in the inner ear.

Hydrogen molecules have recently been reported to act as an antioxidant that selectively reduces hydroxyl radicals, and have been shown to decrease cerebral infarction volume after ischemia [11]. Hydrogen gas also shows protective effects in hepatic injury [12], myocardial infarction [13], and glucose metabolism in patients with type 2 diabetes [14]. In the cochlea, Kikkawa et al. reported that hydrogen gas alleviated ROS-induced ototoxicity [15]. We thus applied their method to vestibular hair cells and investigated hair cell function. The function of hair cells was monitored by accumulation of FM1-43, which is known to reflect the activity of mechano-electrical transducer (MET) channels [16]. We also evaluated the generated hydroxyl radicals by fluorescence emission of 2-[6-(40-hydroxy) phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) to examine the protective mechanisms.

Material and methods

Animals

ICR mice at 2 postnatal days (P2) were used in this study and were purchased from Shimizu Experimental Animals (Hamamatsu, Japan). The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols.

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Vestibular maculae explant culture

P2 ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected and the vestibular maculae were freed from the surrounding tissue and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After removing the otoconia, samples were placed on 12 mm collagen-coated cover glasses (4912-010, Iwaki, Asahi Glass Co. Ltd, Tokyo, Japan), followed by culture in serum-free modified Eagle's medium (MEM; Invitrogen, Eugene, OR, USA) supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako), for 24 h at 37°C in humidified (95%) air:5% CO_2.

Antimycin A application and hydrogen treatment

Explants were incubated in medium containing antimycin A (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 1 μg/ml. Cultures were maintained for 24 h. At the same time, explants were cultured initially in an airtight box (Chopla Industries, Inazawa, Japan) with reduced CO_2-dependence media, MEM, and Leivovitz's L-15 medium (Invitrogen, CA, USA) mixed in a 1:1 ratio [14], supplemented with 3 g/l glucose and 0.3 g/l penicillin G, at 37°C in humidified (100%) atmospheric air. After 24 h, the medium was changed to one containing antimycin A at a concentration of 1 μg/ml, with or without hydrogen gas for another 24 h. Hydrogen gas was dissolved directly into the media, and a high content of dissolved hydrogen (1.3 ± 0.1 mg/l) was confirmed using a hydrogen electrode (Model M-10B2; Able Corporation, Tokyo, Japan). The pH of the culture media without hydrogen gas was 7.18 ± 0.02, and that of culture media with hydrogen gas was 7.52 ± 0.02. The prepared media were used for culture within 30 min.

Immunohistological analysis

At the end of the culture period, samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then provided for immunostaining for myosin VIIa to evaluate the number of surviving hair cells. Specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA, USA). Alexa-Fluor 568 goat anti-rabbit IgG (1:100; Invitrogen, CA, USA) was used as the secondary antibody. Specimens were then incubated in Alexa-Fluor 488-conjugated phalloidin (1:100; Invitrogen) to label F-actin. Specimens were examined with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify hair cell loss after treatments, hair cells were counted in more than three regions of vestibular epithelia.

Counting of remaining hair bundles

Remaining hair bundles were measured using a 10 × 40 eyepiece reticule. Each square of the reticule was 100 μm on each side. Remaining hair bundles were counted in each of three randomly selected fields containing both striolar and extrastriolar regions, and the values obtained were averaged. At least 10 vestibules were examined for each set of conditions.

FM1-43 accumulation of hair cells

A lipophilic dye, FM1-43 FX (Invitrogen), which has been shown to enter hair cells through transducer channels [16], was used to detect hair cells with active transducer channels by accumulation of FM1-43FX (5 μM FM1-43 in PBS, prepared from 10 mM stock solution in DMSO). The mechano-electrical transduction activity of hair cells was quantified based on dye accumulation by applying mechanical stimulation for 10 s [16]. Subsequently, explants were washed three times with PBS for 10 min each, followed by fixing with 4% PFA. FM1-43 accumulation within the cells was observed under a fluorescence microscope.

HPF analysis

At the end of the experiments, explants were treated with 30 mM HPF (Daiichi Pure Chemicals Co., Tokyo, Japan) for 20 min to detect cellular hydroxyl radicals.

Fluorescent images were captured with a Leica TCS-SP2 confocal microscope. All images were taken under the same laser intensity, detector gain, and offset values.

Statistical analysis

Quantitative differences were evaluated by two-way factorial analysis of variance (ANOVA). Significance was evaluated at a level of $p < 0.05$. Data are presented as means and standard errors, along with the number of explants under each condition.