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ORIGINAL ARTICLE

Assessment of in Vitro and in Vivo Transfection Efficiency of the Biodegradable Polymer Chitosan in the Inner Ear

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Background: Sensorineural hearing loss is a significant problem worldwide and a condition that is not completely cured by currently available therapy. Gene therapy of the inner ear offers an exciting alternative and it has been suggested that this therapeutic modality could be used in treatment aiming at preventing, reversing or managing cochlear disorders. Because of their desired properties as an alternative to the viral vectors, non-viral vectors have been extensively explored for gene delivery. One example is chitosan, a biodegradable cationic polymer.

Objective: To evaluate the in vitro and in vivo transfection efficiency of chitosan as a non-viral gene carrier for gene delivery to cells of the inner ear.

Materials and Methods: Organotypic cultures of the hearing organ, the organ of Corti, were prepared from postnatal day 2 rats, and exposed to chitosan carrying plasmid DNA (pDNA) encoding for green fluorescent protein (GFP) for 24-48 hours. The in vivo transfection efficiency was tested at two time points, at one day or seven days after infusing chitosan/pDNA polyplexes through osmotic pumps into the cochlea of adult guinea pigs (n=41). The tissue was then processed for anti-GFP immunostaining (in vitro and in vivo) and RT-PCR (in vivo).

Results: The in vitro assessment showed prominent GPF transfection after 24-48 hours, while the in vivo GFP transfection in the inner ear was inconsistent and did not show good correlation with the in vitro transfection.

Conclusion: It can be concluded that the using chitosan as a carrier for the in vivo transfection, is associated with varying and in consistent degree of transfection.

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Introduction

Cochlear gene therapy is a novel and potential treatment option for inner ear disorders. In mammals, the loss of the inner ear hair cells is permanent as they do not regenerate. However, gene therapy has been suggested as a method for preventing, arresting, reversing or curing hearing disorders by expression of therapeutic genes. Several factors combine to make gene therapy attractive and suitable for treating inner ear disorders. These include the relative isolation of the cochlea from the surrounding structures thus minimising unwanted effects on other tissues, the limited direct blood supply of the cochlea reduces the risk of a possible immune response, and as the cochlea is fluid-filled, all of the functionally important cells are easily reached which facilitates a wide transfection of the system [1-3].

An efficient delivery system for delivering transfecting genes into target cells is essential for successful gene therapy, and a prerequisite for future clinical application. Several techniques of gene delivery into inner ear have been developed; e.g. miniosmotic pump infusion or microinjection into scala tympani through the round widow membrane or a cochleostomy, and direct application to the round window membrane [4]. In order to have an efficient transfection, the DNA must interact with blood cells, vascular endothelial cells and with immune system. However the naked DNA is rapidly degraded by plasma nucleases and moreover, as the DNA is negatively charged it passes through the negatively charged cellular membranes with difficulty [5,6]. Hence, for effective clinical use of cochlear gene therapy, an effective delivery system and a proper vector providing both protection and

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efficient delivery of the DNA is of great importance. A variety of viral and non-viral gene vectors have been developed for the delivery of the genes. Although viral vectors have clear advantages, e.g. providing rapid and stable transfection, their use is limited due to safety concerns and drawbacks such as immunogenicity and potential oncogenic effects. Thus non-viral gene delivery vectors have been suggested as safe alternatives with several advantages including the ability to transfer genes of unlimited size, solubility in body fluid, minimal host immune response, storage stability, and their cost efficiency. On the other hand, non-viral vectors have generally lower transfection efficiency compared to viral vectors, and provide shorter period of gene expression [4,7-9].

Liposomes, cationic lipids, and polymers are presently the most commonly used non-viral vectors. Liposomes induce strong gene expression, but the liposome/DNA complexes are unstable in vivo [10]. Cationic lipids are positively charged, and thus interact with the negatively charged DNA to form complexes that protect DNA against nuclease degradation. However, toxicity and the relatively low transfection efficiency are main disadvantages that limit their potential use. Encapsulation of plasmid DNA (pDNA) in biodegradable polymers potentially offers a way to protect the pDNA from degradation [11] while providing controlled pDNA release [12]. DNA/polymer complexes are more stable than with cationic lipids [5,6].

Chitosans have unique properties that make them attractive for drug and non-viral gene delivery [8,13-15]. Chitosans form polyelectrolyte complexes with DNA and have been successfully used as a non-viral gene delivery system both in vitro and in vivo [8,12,16,17]. Chitosan, the only natural positively charged polysaccharide is a biodegradable polysaccharide polymer of N-acetyl-D-glucosamine and D-glucosamine obtained by the alkaline deacetylation of chitin, a polysaccharide found in the exoskeleton of crustaceans and insects[18]. Chitosan enhances the transport of drugs and large molecules across the cell membrane due to its mucoadhesive property that permits a sustained interaction between the macromolecule being “delivered” and the membrane. It has also the ability to open intercellular tight junctions, facilitating transport into the cells [16]. The trisaccharide substituted chitosan oligomers possess higher gene transfer efficacy compared with unsubstituted oligomers [17,19]. Combination of AAM substitution and branching of the backbone of the chitosan allow for the preparation of sterically stabilized DNA nanoparticles. The self-branched glycosylated trisaccharide-substituted chitosan oligomers (SB-TCO) is fully soluble at a neutral pH and forms physically stable DNA nanoparticles without impairing the intracellular release of DNA[17]. The purpose of the current study was to evaluate whether expression of a reporter gene can be induced in cochlear cells using chitosan (SB-TCO) as a carrier both in vitro and in vivo.

Materials and Methods

1 - Experimental design

In the *in vitro* experiments, twenty postnatal day 2 Sprague Dawley rat pups (Harland, the Netherlands) were used. For the *in vivo* experiments, adult albino guinea pigs (n=41; body weight 250-330 g; Lidköpings Kaninfarm, Lidköping, Sweden) were used. The animals were divided into 3 groups according to the type of treatment; chitosan/pDNA polyplexes group (n=15), pDNA group (n=14), and chitosan group (n=12). All animal handling and experimental procedures were performed in accordance with ethical standards of Karolinska Institutet, and consistent with national regulations for the care and use of animals (ethical approvals N347/05, N35/07, N342/07, N32/07 and N13/10).

1.1 In vitro tests: Organotypic cultures of organ of Corti

Rat pups were sacrificed at postnatal day 2 and the cochleae were carefully removed from the skull in phosphate-buffered saline (PBS) under microscope (Zeiss). The cochleae were then placed in PBS supplemented with 5.5 ml/ ml of 30% glucose. The stria vascularis, spiral ligament and spiral ganglion were carefully pulled away from the organ of Corti. Then 4-5 explants per group were transferred to culture inserts (Millicell- CM 0.4 mM 30 mm diameter; Millipore) placed in 6-well plates and cultured in 750 µl DMEM, (Gibco, Invitrogen) supplemented with 10 ml/ ml of N1 supplement (Sigma); 5.5 ml/ ml of 30% glucose (Sigma) and 100 units/ml penicillin (Gibco, Invitrogen). After 24 hours of incubation at 37°C and 5% CO2, the cultures were exposed to chitosan/pDNA polyplexes (750 µl) for 24 or 48 hours. Following 24 hours incubation with chitosan/pDNA polyplexes, the media was replaced by 750 µl of fresh culture medium in the 24 hours exposure group and were further incubated for another 24 hours but in the 48 hours exposure group the

cultures were left all 48 hours with chitosan/pDNA polyplexes. Thus the growing time was the same in all cultures (48 hours). The cultures were grouped as follows: (1) cultures exposed to chitosan/pDNA polyplexes for 24 hours, and (2) cultures exposed to chitosan/pDNA polyplexes for 48 hours. Control cultures, containing only fresh culture media, were run concurrently with the experimental cultures. The GFP expression was measured by recording of fluorescence intensity using fluorescence microscopy.

1.2 In vivo experiments

Adult albino guinea pigs were anesthetized with xylazine 5 mg/kg and ketamine 40 mg/kg i.m. Local anesthesia with 0.25% bupivacaine was used along areas to be incised and over the mid-line of the back. A heating pad (Kanthal, type 3654 M) kept the body temperature between 36.5 and 37.5°C. Doxycycline (Doxyferol ® 1 mg/kg) was administered i.p. as a prophylactic antibiotic. After shaving and disinfecting the skin with iodine and 70% alcohol, a post-auricular incision was made to expose the bulla. Under an operating microscope, a hole was made in the bulla, to identify the basal turn of the cochlea and round window membrane (RWM). A small hole was drilled in the basal turn of the cochlea, and a cannula (inner diameter 0.72 mm; Scientific Commodities Inc.) connected to a miniosmotic pump (Alzet®mini-osmotic pump, either # 1007D; flow rate 0.5µl/h or pump # 2001D; flow rate 8µl/h) was inserted into scala tympani [20]. The cannula was secured at the bulla by a small drop of tissue adhesive (Histoacryl, Aesculap AG, Germany). Dental cement (Dentalon plus Heraeus Kuzler, Inc. NY, USA) was used to cover the hole in the bulla and secure the cannula in place. The mini-osmotic pump was placed under the skin on the back of the animal; containing 100-200 µl (depending on the duration of treatment and model of the pump) of chitosan, pDNA or chitosan/pDNA polyplexes, then the skin was closed in layers. Only the left cochleae were infused whereas the right cochleae were used as a control. At the end of surgery, the animal received 5 ml of body warm saline s.c. to prevent dehydration, and buprenorphine hydrochloride (Temgesic® 0.05mg/kg) s.c. as an analgesic agent. The well-being of the animals was kept under control and their weight was carefully monitored for a few days after surgery. One and 7 days later, the animals were sacrificed using an overdose of sodium pentobarbitone i.p. and the temporal bones containing the cochleae rapidly removed. The tissue was then processed either for immunohistochemistry or PCR. Details of the experimental design, duration of treatment, and methods used to process the samples are shown in Figure 1.

2 - Preparation of chitosan/pDNA polyplexes

Reporter plasmid (gWiz™GFP) containing a cytomegalovirus promoter (CMV) and green fluorescent protein (GFP) was purchased from

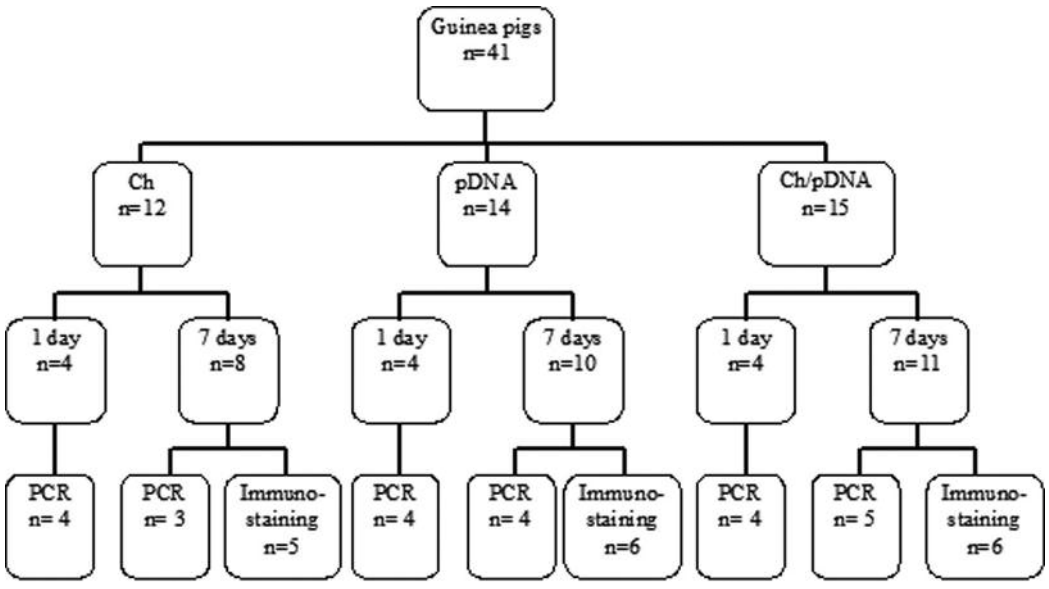

Figure 1. Overview of the experimental design of the in vivo experiments, showing the number of animals used in different groups (chitosan alone [Ch], plasmid DNA [pDNA] and chitosan/pDNA polyplexes [Ch/pDNA]), duration of treatment, and processing methods.

Aldevron, Fargo, ND, USA. A fully characterized self-branched and trisaccharide-substituted chitosan oligomer (SB-TCO) was used in this study. The chitosan was prepared by controlled nitrous acid depolymerization of fully de-N-acetylated chitosan (Fraction of acetylated units, F_A<0.002) followed by self-branching and substitution with the trimer 2-acetamido-2-deoxy-D-glucopyranosyl-ß-(1-4) 2-acetamido-2-deoxy-D-glucopyranosyl-ß-(1-4)-2,5-anhydro-D-mannofuranose (A-A-M) as described previously [17]. The molecular weight (M_w: 22000) and molecular weight distribution (M_n: 13700) were analyzed by Size-Exclusion Chromatography with refractive index (RI) and a Multi-Angle Laser Light Scattering detector (SEC-MALLS). The Polydispersity index (PDI) was 1.61 and the degree of substitution of A-A-M (7.3%) was determined by nuclear magnetic resonance spectroscopy 1H NMR.

Polyplexes of chitosan and pDNA with amino/phosphate (A/P) ratios of 30:1and pDNA were prepared by the self-assembly method. For the in vitro experiments, the polyplexes were prepared as described above with a pDNA concentration of 13.3 µg/ml. The polyplexes were further diluted 1:2 in OptiMEM I (Gibco, Invitrogen) supplemented with 270 mM mannitol and 20 mM HEPES to adjust the osmomolarity and pH of the formulation. For the in vivo experiments, an aliquot of 0.264 ml of pDNA (0.5 mg/ml) was diluted in deionized water (MQ-Millipore) and a required amount of sterile filtered chitosan stock solution in MQ-grade water (4 mg/ml) was added during intense stirring on a vortex mixer (1200 rpm, Heidolph REAX 2000, Kebo Lab, Sweden), yielding a pDNA concentration of 110 µg/ml. The size of chitosan/pDNA polyplexes, expressed as mean hydrodynamic diameter (z-average), was 128 ± 7 nm as determined by dynamic light scattering on a Nanosizer ZS (Malvern Instr., Malvern, UK).

3 - Immunohistochemistry

In vitro experiments: The cultures were rinsed in phosphate buffered saline (PBS) and fixed for 1 hour in 4% paraformaldehyde in PBS while still on the culture membranes. The culture explants were rinsed in PBS, removed from the membranes and processed for immunohistochemical staining. The tissue was permeabilized with 0.3% Triton X-100 in PBS for 10-20 min at room temperature then blocked for 1 hour with 5% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100 to block unspecific binding sites. Afterwards cultures were incubated overnight with rabbit polyclonal anti-myosinVIIa (1:1000 diluted in blocking solution, Proteus Bioscience) and goat anti GFP (1:500, Abcam) overnight at 4°C. On the following day, cultures were washed and incubated with secondary antibodies, Cy3 goat anti rabbit IgG (1:400, Jackson ImmunoResearch) and alexa fluor 488 donkey anti-goat IgG (1:400, Molecular Probes). Finally the cultures were stained with DAPI and mounted in water soluble mounting media (Mowiol, Calbiochem).

In vivo experiments: After decapitation and removal of the temporal bone, the left cochlea was immediately dissected out and immersed in 4% paraformaldehyde in PBS. Under the microscope, a small fenestration was made in the apex, and the round window membrane (RWM) was perforated to allow the fixative to reach the entire organ of Corti. One hour later the samples were transferred to 0.5% paraformaldehyde and stored at 4ºC until further processing. The samples were decalcified in EDTA solution (0.1M ethylenediaminetetraacetate) for 2 weeks. After decalcification, the tissue was cryoprotected in a 10% sucrose solution followed by a 30% sucrose solution (each for 24 hours) at 4°C and embedded in OCT (Tissue Tek, CA, USA), frozen in super cooled isopentane and stored in the freezer until being sectioned (14 µm) using a cryostat (Leica CM 3050S) and mounted onto super-frost glass slides (Thermo Scientific). The sections were re-hydrated in PBS before the immunostaining. To block non-specific binding sites, sections were incubated in PBS containing 10% NGS, 5% BSA and 0.3% Triton-X, (blocking solution) for one hour at room temperature. The sections were then incubated over night with primary antibodies for GFP, chicken anti-GFP polyclonal antibody (1:1000, Chemicon), and after three washes with PBS with a secondary antibody fluorescein (FITC) labelled goat anti-chicken IgY, (1:200, Aveslab) for one hour. Finally, sections were washed three times in PBS before mounting in (Mowiol, Calbiochem) and cover slipped. The sections were examined using fluorescent microscopy (Zeiss).

4 - RT-PCR analysis

The temporal bones (n=26 samples) were opened and immersed in RNA later solution (Qiagen cat. no.76106) for further dissection. The RWM and apical turn were fenestrated. Soft parts of the cochlea

including the organ of Corti, lateral wall and modiolus were isolated under the microscope and transferred to a tube containing 1ml of ice-cold TRIzol (Invitrogen, Carlsbad, CA, USA) and homogenized thoroughly with a pestle, and samples were stored overnight at -20°C. Total RNA was isolated using TRIzol reagent and RNeasy® MinElute Cleanup kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. To eliminate possible DNA contamination, samples were subjected to on-column DNase digestion with DNase I (Qiagen®; cat. no.79254), according to the manufacturers protocol. Final RNA concentration was measured using Invitrogen Qubit®.

One-Step RT-PCR was performed using SuperScript III kit (Invitrogen) on all samples. The contents of a 10-µl RT-PCR reaction were 5 µl of 2X Reaction mixture buffer, 1ml template RNA, 0.4 µl of sense and anti-sense primers (10 µM), 2.8 µl RNase free water and 0.4 µl of SuperScriptIII RT/ Plat TaqMix. Optimal annealing temperature and PCR cycle number for the primers were determined with a gradient thermocycler (PTC-200, MJ research).The reverse transcription (cDNA) synthesis was performed in one cycle of 50°C for 30 min.

The PCR amplification consisted of 30 cycles of 15 seconds denaturation at 94°C, 30 seconds at 59°C annealing temperature and one minute of extension at 68°C. A final single extension cycle of 68°C for 5 minutes was added at the end. No-RT controls were run for each sample by replacing the RT/ TaqMix with a DNA polymerase (Invitrogen). RT-PCR products were run on 2% agarose gels containing ethidium bromide (0.5 µg/ml) and visualized in ultraviolet light. A One-Step RT-PCR with GAPDH specific primers was performed to verify RNA integrity. The cDNA synthesis was performed in one cycle of 55°C for 30 minntes. The PCR amplification consisted of 33 cycles at of 15 seconds denaturation at 94°C, 30 seconds at 58°C annealing temperature and one minute of extension at 68°C. A final single extension cycle of 68°C for 5 minutes was added at the end. The content in the reaction tubes was as described above. The primers used were designed using online free software Primer3 [21] and were as follows: GFP forward primer (GCCCGAAGGTTATGTACAGC) and reverse primer (GTCCCAGAATGTTGCCATCT).

GAPDH forward primer (GCCAACATCAAGTGGGGTGATG) and reverse primer (GTCTTCTGGGTGGCAGTGATG).

Results

1 - *In vitro results: organotypic cultures*

Organ of Corti cultures prepared from postnatal day 2 rats were incubated with chitosan/pDNA polyplexes for 24 or 48 hours. Immunostaining of the cultures revealed GFP expression in cultures treated with chitosan/pDNA polyplexes (Figure 2 A-F) which was not observed in control cultures (Figure 2 G-I). Cells in the region of the inner hair cells (IHCs) and the outer hair cells (OHCs) were transfected, and expressed GFP (Figure 2A and D). The cells were not transfected equally and expression was observed in some regions of the cultures. Additionally, more transfected cells were observed in the cultures incubated for 48 hours (Figure 2 D) compared to 24 hours (Figure 2 A).

2 - *In vivo results – guinea pig model*

The feasibility of chitosan mediated gene delivery was tested in vivo by direct cochlear infusion using osmotic pumps. To detect and evaluate transfection, the samples were processed for immunohistochemistry (Figure 3 A-H) and RT-PCR (Figure 4 A-C).

Following in vivo administration of chitosan/pDNA polyplexes, the samples were processed for immunostaining and the spiral ganglion and organ of Corti were examined for signs of GFP expression. Treated samples were compared with corresponding areas in control samples with and without using primary antibodies. There were no differences between sections treated with chitosan/pDNA polyplexes, pDNA, and chitosan alone as seen in Figure 3 A-H.

As the immunostaining results were negative, different runs of RT-PCR were performed on the samples treated with chitosan /pDNA polyplexes (n=9), pDNA (n=8) or chitosan alone (n=7) for either one or seven days, and control samples (n=2), Figure 4 A-C. The results showed varying degree of GFP expression as four out of 9 samples treated with the chitosan/pDNA polyplexes showed positive transfection (Figure 4 A-B). The sample that showed the most intense band originated from an animal receiving chitosan/pDNA polyplexes for 7 days (Figure 4 A, lane 6). However, the overall transfection pattern was inconsistent, e.g. in Figure 4 A, lanes 5-7 and B, lanes 7 and 8. These are replicates of same treatment group (chitosan/pDNA polyplexes for 7 days) but showed varying degree of transfection with no transfection in 3 samples (Figure 4A, lane 5 and B, lanes 7 and 8). The absence of a band in these samples most probably was due to absence of transfection as all samples were tested for RNA

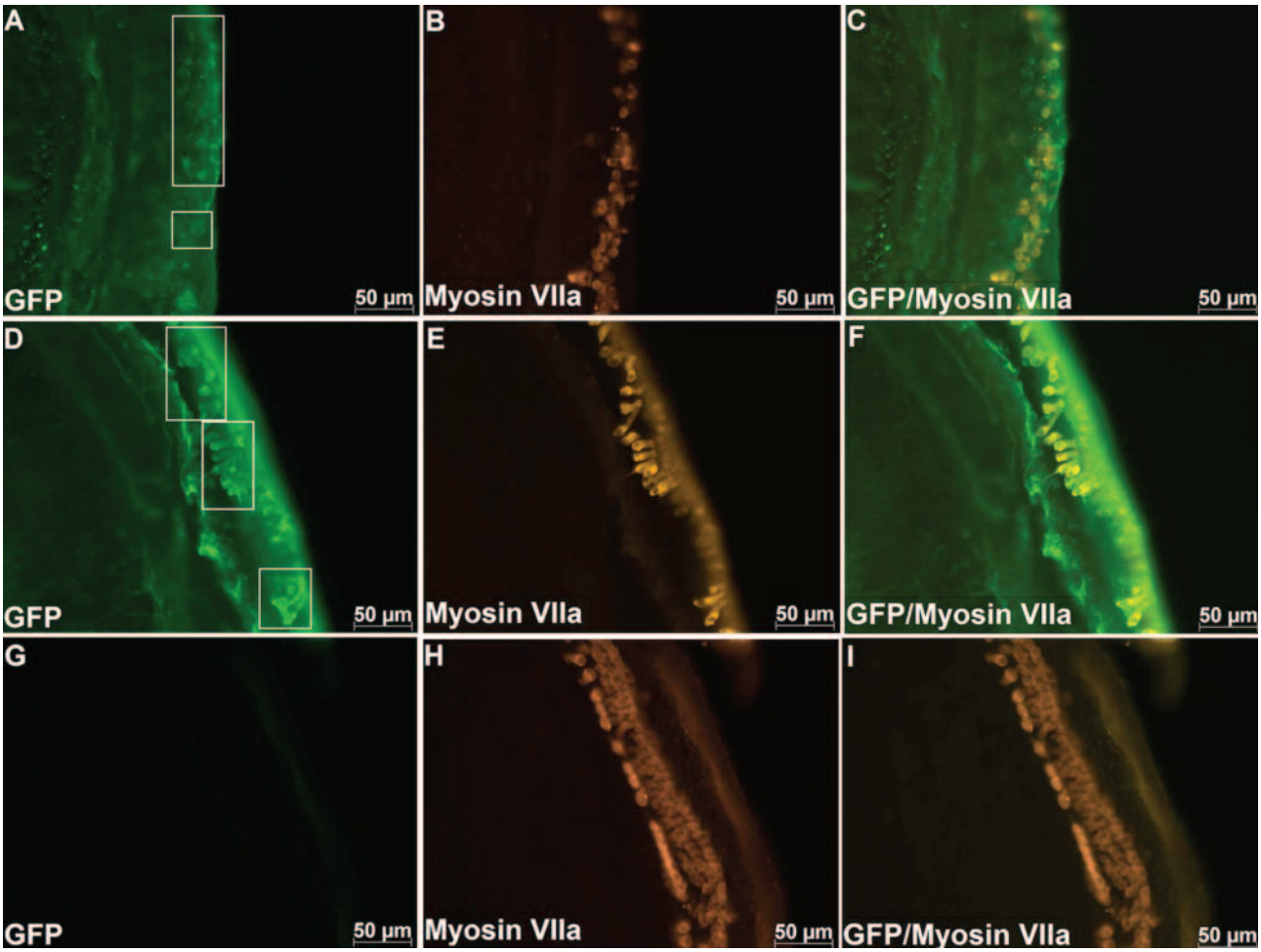

Figure 2 A-I. In vitro fixed organotypic culture of postnatal day 2 rat transfected with Chitosan/pDNA, stained for GFP, Myosin VII a, and merged together respectively examined with fluorescent microscope (magnification 40 X). The areas of GFP expression are marked. Upper panel **(A-C)** shows the cultures of organ of Corti 24 hours after transfection. Middle panel **(D-F)** shows the cultures 48 hours after transfection. Lower panel **(G-I)** shows control cultures.

integrity. The same inconsistency in results was observed for replicate samples of animals receiving chitosan/pDNA polyplexes for 1 day; transfection in 2 samples (Figure 4 A, lanes 8 and 9) and no transfection in another 2 samples (Figure 4 B, lanes 5 and 6). Samples treated with pDNA for 1 and 7 days showed inconsistent results (Figure 4 A, lanes 3, 10 and 11, B, lanes 3 and 4, and C, lanes 1, 2 and 4). Samples treated with chitosan alone (Figure 4 A, lane 4, B, lanes 1 and 2, and C, lanes 3 and 5-7) showed no bands.

Discussion

In the current study, we wanted to investigate the efficiency of chitosan as a pDNA vehicle for gene delivery into the cells of the inner ear. To the best of our knowledge, this study represents the first study to use chitosan for gene delivery into the inner ear both in vitro and in vivo. In the in vitro experiments, both inner hair cells (IHCs) and the outer hair cells (OHCs) displayed expression, confirming successful pDNA delivery in vitro. However, not all cells were transfected equally and they showed different GFP intensity in cultures incubated with the transfection medium for 48 hours (Figure 2 D) compared to 24 hours exposed cultures (Figure 2 A). Following 24 hours incubation with chitosan/pDNA polyplexes, the media was replaced by fresh culture medium in the 24 hours exposure group and were further incubated for another 24 hours but in the 48 hours exposure group

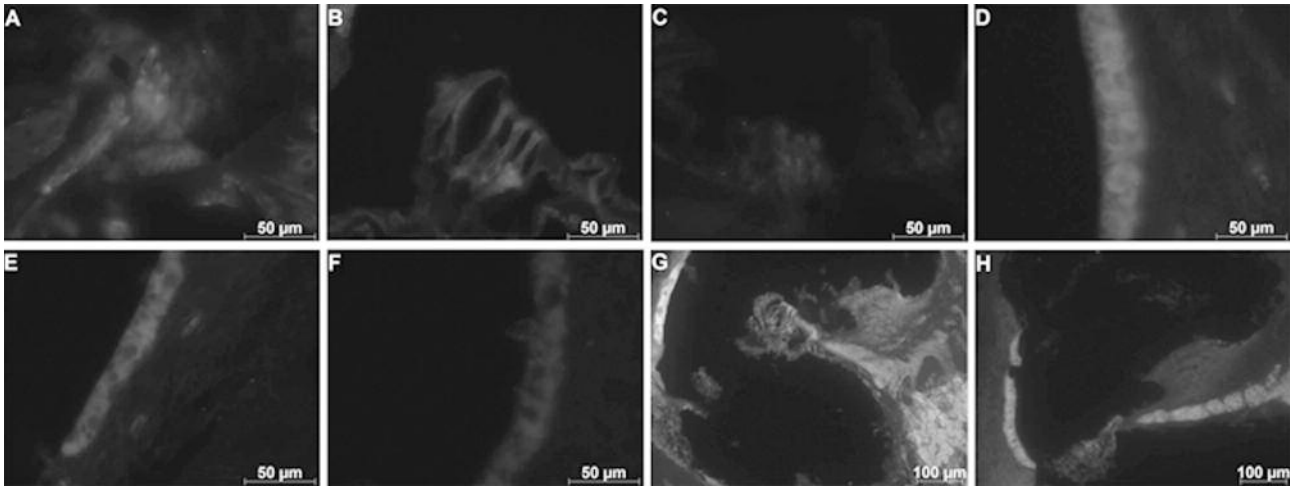

Figure 3 A-H. In vivo immunostained sections of the organ of Corti and lateral wall are seen. Sections of organ of Corti not treated with antibody against GFP **(A)** Chitosan/pDNA polyplexes, **(B)** pDNA, and **(C)** Chitosan alone (magnification 40 X). Sections of the lateral wall region (stria) not treated with antibody against GFP **(D)** Chitosan/pDNA polyplexes, **(E)** pDNA, and **(F)** Chitosan alone (magnification 40 X). Sections of organ of Corti treated with antibody against GFP **(G)** Chitosan/pDNA polyplexes and **(H)** Chitosan alone (magnification 20 X).

the cultures were left all 48 hours with chitosan/pDNA polyplexes. Thus the growing time was the same in all cultures (48 hours). It is possible that with the prolonged incubation time (48 hours) more time had elapsed for protein translation leading to increased expression. The in vivo transfection in the inner ear did not show good correlation with the in vitro transfection. With the immunostaining, no specific expression of GFP was seen in sections treated with chitosan/pDNA polyplexes, indicating no transfection of GFP or at least no translation of the gene (cf. figure 3 A, D and G). With RT-PCR analyses, the results showed varying degree of GFP expression in replicates of same treatment group (cf. Figure 4 A-C). Despite these inconsistent results of the in vivo experiments, the results of the present study suggest that the chitosan/pDNA polyplexes are capable of transfecting cell in the cochlea and organ of Corti in vitro and to some extent in vivo. The differences in expression levels might be due to the differences in efficiency with which the GFP gene was expressed within the cell. Variable transfection efficiencies may happen because of differences in the promoter used to drive transgene expression [22]. However, the CMV promoter used in this study is expected to drive high levels of GFP reporter sequence in a variety of cell. The results of the in vivo experiment were not completely consistent with those of in vitro experiments. This could be partially due to direct application of the chitosan/pDNA polyplexes in the transfection medium to the cultures in the in vitro experiments. These differences in transfection between in vivo and in vitro experiments might also be due to the exposure sites (scala tympani in the in vivo while the perilymphatic and endolymphatic spaces in the in vitro) and the administered amount of chitosan/pDNA polyplexes. Luebke and co-worker stated that the adenoviral titers for in vivo transfection should be 100× higher than for in vitro adenoviral infections [22]. In our study, the pDNA concentrations were 13.3 µg/ml and 110 µg/ml in the in vitro and in vivo experiments respectively. The transfection media were 750 µl in the in vitro experiments and 100-200 µl in the in vivo experiments depending on the type of the osmotic pumps used. Preparation of higher concentration of DNA nanoparticles would have resulted in immediate aggregation. Another possible cause for the inconsistent transfection might be the aggregation of nanoparticles in vivo. It is very typical for all cationic nanoparticles to aggregate partially at physiological condition, and these aggregate are too large to be taken up. This could further reduce the amount of available pDNA.

The absence of transfection of the GFP in the inner ear in vivo in the present study may be related to some of the factors which have been previously described by Borchard that negatively influence transfection process such as the efficiency of targeting the cells, the ability to cross the cell membrane and to enter the nucleus [13]. The means and route of gene delivery to the cochlea is complicated by its structural complexity,

compartmentalized nature of isolated endolymphatic and perilymphatic compartments, and sensitivity of the hair cells to trauma [23]. Despite the fact that in the in vivo experiments we used an osmotic pump infusion as a prolonged and sustained delivery method, the results were variable; no expression with the immunostaining (cf.Figure 3 A-H) and inconsistent expression levels with PCR analyses (cf. Figure 4 A-C). The variability may be due to the pDNA not reaching the target cells facing the endolymphatic fluid within scala media. Tan and co-worker suggested that there is no dissemination of transfecting agent from the perilymphatic to the endolymphatic fluid space especially when using cationic polymer–DNA complexes [9]. Contacts between the transfecting agent and regions of transfection are important to get successful transfection.

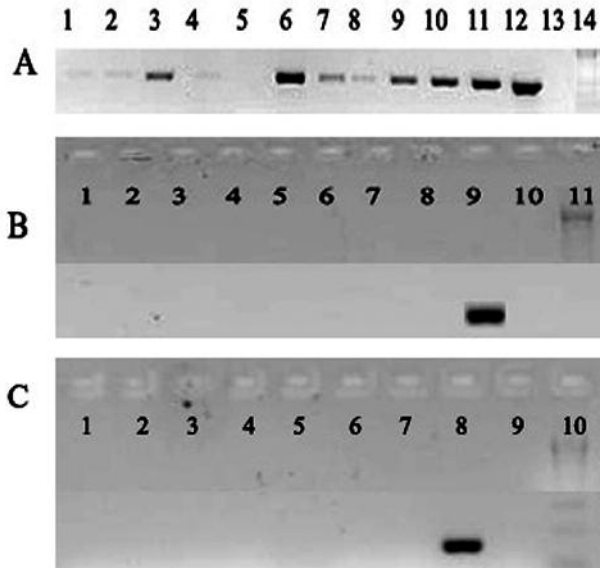

Figure 4 A-C. The electrophoresis of One-Step RT-PCR products on agarose gel is seen. **(A)** Control samples (lanes 1 and 2), pDNA after 7 days (lane 3), chitosan after 7 days (lane 4), chitosan/pDNA polyplexes after 7 days (lanes 5-7), chitosan /pDNA polyplexes after 1 day (lanes 8 and 9), pDNA after 1 day (lanes 10 and 11), positive and negative controls (lanes 12 and 13), and DNA size marker (lane 14). **(B)** chitosan after 1 day (lane 1), chitosan after 7 days (lane 2), pDNA after 1 day (lane 3), pDNA after 7 days (lane 4), chitosan/pDNA polyplexes after 1 day (lanes 5 and 6), chitosan/pDNA polyplexes after 7 days (lanes 7 and 8), positive and negative controls (lanes 9 and 10) and DNA size marker (lane 11). **(C)** pDNA after 7 days (lanes 1 and 2), chitosan after 7 days (lane 3), pDNA after 1 day (lanes 4), chitosan after 1 day (lanes 5-7), positive and negative controls (lanes 8 and 9) and DNA size marker (lane 10).

Conclusion

It can be concluded that using chitosans as a carrier for the cochlear gene delivery have resulted in transfection of the cochlear tissue in vitro. The in vivo experiment, showed variable transfection results and were inconsistent with the in vitro results.

Acknowledgments

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