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Review article

Ultrasound targeted microbubble destruction-mediated gene delivery system: application to therapy for ocular disease

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Background: Ocular disorders have greatest potential for benefit from gene therapy. The major obstacle in the clinical application of gene therapy is not due to the lack of an ideal gene, but rather the lack of a clinically safe and efficient gene transfer method. Ultrasound (US) targeted microbubble destruction (UTMD)-mediated gene delivery system as a noninvasive gene transfer method is now widely used in gene therapy of cardiovascular disease, muscular tissue, and tumor, and proved to effectively enhance gene transfer in various studies in vitro and in vivo. However, it is just the beginning of application for ophthalmological disease.
Objective: Review the latest advancements in UTMD-mediated ocular gene transfection and discuss mechanisms of UTMD involved in gene transfection, obstacles, and limitations to the use of this technology, as well as the perspectives for future applications of UTMD-mediated gene delivery system.
Methods: Summarize published literature concerning UTMD-mediated ocular gene transfection.
Results: UTMD is an effective and safe gene delivery method of therapy for ocular diseases. Considerable progress has been made in US or UTMD-mediated viral and nonviral ocular gene delivery to retina, like recombinant adeno-associated virus (rAAV) and nanoparticles as nonviral gene carriers. In addition, UTMD has potential for producing the blood-retinal barrier opening and serves as a promising method for intravenous ocular gene delivery.
Conclusion: UTMD-mediated gene delivery system could effectively enhance gene transfer into ocular tissue. Though several problems remain to be solved, UTMD is a promising technology for the targeted gene therapy of ocular disease.

Keywords: Gene delivery, ultrasound, ocular disease, microbubbles

Many hereditary and acquired ocular diseases have the great potential for benefit from gene therapy [1]. Gene transfer into ocular tissues has been demonstrated with growing functional success. This may develop into a new therapeutic tool for clinical ophthalmology. The major obstacle in the clinical application of gene therapy is not due to the lack of an ideal gene, but rather the lack of a clinically safe and efficient gene transfer method. The basic technology of gene delivery can be divided into two categories, a virus vector–mediated method and a non-virus vector-mediated method [2-4]. The virus vector-mediated method can transfer the gene of interest with higher efficiency, but concern about safety issues prevents clinical application for common disease [4-6]. The non-virus vector-mediated method is comparatively safe, but gene transfer efficiency does not reach a satisfactory level [7-10]. A new delivery method for gene therapy is required to improve the safety and efficiency of viral and non-viral vector infection.

Ultrasound contrast agents (UCAs), in the form of gas-filled microbubbles with a diameter of 1-7 μm, are becoming popular in perfusion monitoring. They are employed as molecular imaging agents. Microbubbles are manufactured from biocompatible materials, and they can be destroyed by ultrasound

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irradiation. This destruction phenomenon can be applied to targeted drug and gene delivery [11]. Ultrasound (US) targeted microbubble destruction (UTMD)-mediated gene delivery system is now widely used in the targeted gene therapy of cardiovascular disease, muscular tissue and tumors, and proved to enhance gene transfer in various studies in vitro and in vivo [12-16]. However, it is just the beginning of application for ophthalmological disease.

Ultrasound contrast agents

In 1968, Gramiak and Shah [17] published the first article on ultrasound contrast agents (UCAs). With the introduction of microbubble contrast agents, diagnostic ultrasound has entered a new era that allows the dynamic detection of tissue flow of both the macro and microvasculature. More recently, such microbubbles have evolved as experimental tools for organ- and tissue-specific drug and gene delivery.

Components of microbubble shell

The microbubble shell is usually comprised of substances such as lipids, albumin, sugar, and macromolecular polymers [18, 19]. Different types of shells confer different characteristics to the microbubble. Lipid-coated microbubbles are less stable than other shell types, but they are easier to form and yield a more echogenic microbubble. In the 1980s, UCAs were coated with an adsorbed layer of saccharide or protein. The albumin-coated microbubbles Albunex and OptisonTM were the first commercially available, FDA-approved contrast agents. SonoVue is another example of an important family of microbubbles whose membrane consists of phospholipids. More recently, protein-shelled microbubbles have been functionalized to carry targeting ligands [20] and genetic payloads [21, 22].

Microbubble gas contents

Many studies have shown that microbubbles containing gases that have low diffusivity and low blood saturation have a considerably greater longevity [23-25]. Hence, an ideal gas should possess characteristics such as low solubility, high molecular weight, high density, high inertia, and high stability. The commonly used gases are, for example, perfluorocarbon gas and sulfur hexafluoride.

Methods to use UCAs for drugs or gene delivery

UCAs are not only effective in ultrasonic imaging but also can be designed as safe vehicles for encapsulating or co-transporting drugs or genes. There are three methods to use UCAs for therapeutic delivery. The two more common applications of this technique are: (i) to co-administer the microbubbles and the bioactive substance together and (ii) to incubate the microbubbles and the therapeutic together for a certain amount of time, before administering the complex to the target site. Drug- or gene-bearing non-echogenic vehicles/vectors can also be made and then co-administered or incubated with acoustically active microbubbles. These techniques allow attachment of the therapeutic to the microbubble shell, either by electrostatic or weak non-covalent interactions. The third methodology to use UCAs as therapeutic delivery agents is (iii) to manufacture the microbubbles de novo, incorporating the gene or drug into the shell or lumen [26].

Mechanism of UTMD-mediated gene transfection

Intravital microscopy observation of the results of in vivo destruction of microbubbles induced by ultrasound was reported by Skyba et al [27]. It was shown that immediate rupture of the microvessels occurred at the location of microbubble destruction, with extravasation of red blood cells in the interstitial space. This observation opened a variety of possibilities in using microbubbles for therapeutic purposes. Normally, plasmid DNA, and pharmaceutical drug carrier particles have difficulty crossing the endothelial layer and escaping from the bloodstream. UTMD-mediated gene delivery creates conditions to help overcome the endothelial lining barrier in the targeted tissue.

The ability of microbubbles to act as cavitation nuclei when destroyed can increase cell membrane permeability, if cells are located in close proximity to microbubbles. Although ultrasound had been proven to increase cell membrane permeability on its own [28], the use of microbubbles has a significant additive effect. Several explanations are offered for this phenomenon called ‘sonoporation’. First, the microjets cause shear stress on the cell membrane and create transient, non-lethal holes in the plasma membrane, through which a drug or gene is able to diffuse [29, 30]. These pores were visualized by scanning electron microscopy [31, 32], and the mechanism of action could be revealed using a high frame rate camera [33]. Second, the generation of intracellular reactive oxygen species, following the application of ultrasound, might

contribute to permeabilization of the cell membrane without affecting the cell viability. The local, transient temperature increase due to the absorption and dissipation of ultrasound energy may also influence phospholipid bilayer fluidity and thus cell permeability [34]. Other scenarios are the involvement of active transport mechanisms, such as endocytosis and phagocytosis in the uptake of microbubbles, and the fusion of lipid-based microbubbles with the phospholipid cell membrane [11].

Application of ultrasound with microbubbles in ocular gene delivery

Compared to most other tissues of the body, the eye is an excellent candidate for gene therapy as it is easily accessible and immune-privileged [35]. In addition, the eye is a small organ, making the transfection of a significant proportion of the cells of interest a realistic possibility [36]. In addition, most target cells for ocular gene therapy are not undergoing cell division, potentially reducing the risk of oncogenesis. More and more scholars begin to introduce UTMD to ocular gene transfection as it could be used to as a safe, efficient, and targeted method for in vitro and in vivo gene delivery. Current researches on ocular gene transfection mediated by UTMD mainly focus on the corneal and retinal disease.

Gene transfer to cornea and conjunctiva mediated by UTMD

In the sphere of ophthalmology, scholars pay much attention to study US or UTMD-mediated drug and gene delivery on the cornea [37-40]. Sonoda et al. [40] investigated the practical efficacy and safety of ultrasound plus microbubble-mediated gene transfer to rabbit cornea, and demonstrated that US with the albumin-coated microbubbles Optison greatly increased green fluorescent protein (GFP) gene transfer to in vivo and in vitro rabbit corneal cells without apparent tissue damage, while US alone enhanced gene transfer slightly. Our previous study also showed that using US in conjunction with commercially available SonoVue could safely increase enhanced GFP (EGFP) plasmid transfer to the mouse cornea in vivo [41]. The expression of EGFP was not detected over the ocular surface when plasmid alone was injected to mouse eye anterior chamber (**Figure 1A**). In the US and SonoVue group, EGFP expression in the cornea increased from 1st day (**Figure 1B**), then decreased from 7th day and vanished at 21st day. EGFP expression in US and SonoVue group was obviously higher than only US exposure group and liposome group (**Figure 1C, D**).

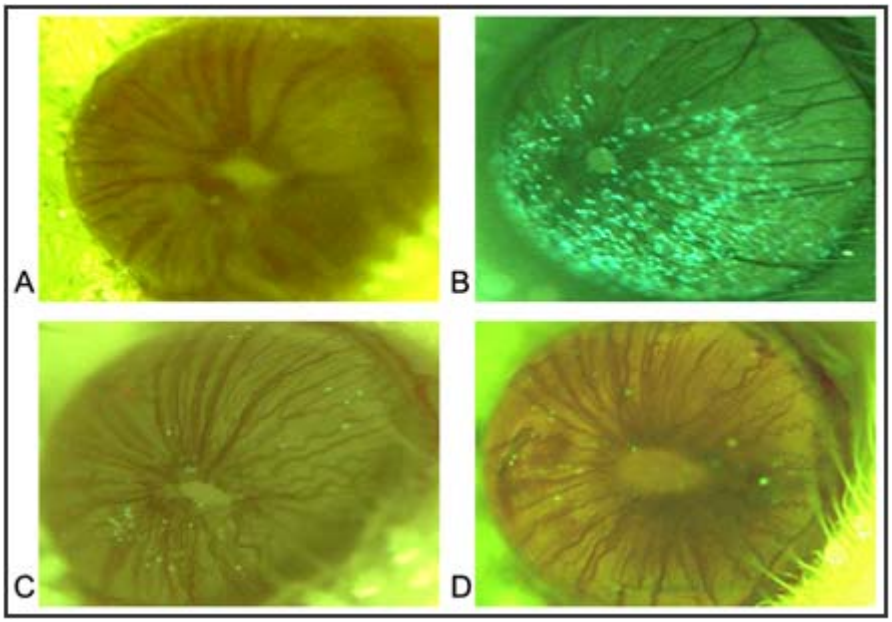

Figure 1. Fluorescence stereoscopic images of EGFP expression at the first day in the cornea after anterior chamber injection of (**A**) EGFP plasmid alone or in combination with (**B**) US and SonoVue, (**C**) US or (**D**) liposome. (40 x magnification).

Another example of a successful gene transfer to ocular surface mediated by UTMD came from a report by Yamashita et al [42]. Using a novel bubble liposome (BL) with ultrasound to deliver GFP to rabbit corneal epithelial cells in vitro and conjunctiva in vivo. BL is composed of polyethylenglycol (PEG) modified liposome containing perfluoropropane gas. It was shown that BL and US exposure significantly increased gene transfer efficacy in cultured rabbit corneal epithelial cells and in rat eyes, strong GFP staining was seen in conjunctiva of BL-US group, which was significantly higher than in any control groups.

Gene transfer to retinal tissue mediated by UTMD

Study on ultrasound and microbubble-mediated gene transfection of retinal ganglion cells (RGCs) has also been carried out [43]. The results showed that UTMD could enhance the GFP reporter gene transfection and expression into RGCs in vitro. The average transfection rate of EGFP with US plus microbubble was 25%. Moreover, transfection of bcl-xl gene mediated by UTMD has an anti-apoptosis effect on the cultured RGCs induced by N-methyl-D-aspartate.

Choroidal neovascularization (CNV) is responsible for most cases of severe visual loss in age-related macular degeneration. Many studies have suggested that the imbalance of angiogenic factor and anti-angiogenic factor expression contributes significantly to the development of CNV. Several research groups have transferred pigment epithelium-derived factor (PEDF) gene, an exceptionally potent inhibitor of angiogenesis, in vivo and verified its inhibitory effect on CNV [44, 45]. However, in their studies, traditional gene delivery techniques, such as liposome based transfection and adenovirus-mediated gene transfer, were used. The traditional gene vectors are unsafe and have low effectiveness. This restricts its application in ophthalmology [46, 47]. Recently, Zhou et al. [48] delivered PEDF gene into retina by UTMD and gene and protein expression of PEDF was detected by quantitative real-time PCR, Western blotting and immunofluorescence staining, respectively. The effect of PEDF gene transfer on CNV was examined by fluorescein fundus angiography. In vitro human retinal pigment epithelial (RPE) cell experiments showed that microbubbles with ultrasound irradiation could significantly enhance the delivery efficiency of PEDF plasmid as compared with microbubbles or ultrasound alone. In the rat CNV model, gene transfection efficiency into the rats' retina mediated by UTMD was significantly higher than that by lipofectamine-mediated gene transfer at 28 days after treatment, suggesting that UTMD was a new, safe, and effective gene delivery technique to transfer gene into the retina. The study also showed that with the administration of ultrasound-mediated microbubbles destruction, the CNV of rats was inhibited effectively.

Gene transfer to retinoblastoma mediated by ultrasound with microbubbles

UTMD-mediated gene delivery to ocular tumors has also been reported. A research group successfully demonstrated that EGFP gene could be transfected into retinoblastoma (RB) cells by using UTMD system and verified that transfection efficiency into RB cells mediated by UTMD was similar to that with lipofectamine 2000 [49]. Although it still lacks in vivo study, this result indicated the feasibility of UTMD-mediated gene therapy to ocular tumors.

Enhancement of virus-mediated gene transfection by UTMD

Currently, the most effective gene therapy vectors are viruses. Early ocular gene therapy experiments used adenoviral vectors, but these were limited by the severity of the inflammatory reaction induced and the relatively short duration for which transgenes could be expressed. Recombinant adeno-associated virus (rAAV) vectors have a number of important advantages over other vectors that make them suitable for transfection studies, in particular the ability to induce long-term transgene expression in the eye and a relative lack of pathogenicity [50-52]. However, the transduction of rAAV occurs with relatively low efficiency, which limits its therapeutic effects. Our recent research outcomes indicate that ultrasound and microbubbles could enhance virus-mediated gene transfection into retinal tissue. Li et al. [53] demonstrated that UTMD could enhance rAAV2 transfection efficiency in human RPE cells in vitro and in Wistar rat retina in vivo as shown in **Figures 2** and **3**. Under the optimal condition, UTMD increased the rAAV2 transfection efficiency in vitro about 74.85%, and the cell viability was above 95%. Similar study was reported by Zheng et al. [54], where the effects of ultrasound or/and microbubbles on rAAV-mediated gene transfection in rat RPE-J cells in vitro

and Wistar rat retina in vivo was accessed. The results found that rAAV2-mediated EGFP expression in vivo was significantly enhanced by ultrasound and microbubbles (**Figure 4**), while rAAV2-mediated gene transfection in vitro was significantly enhanced by ultrasound or microbubbles alone, but not their combination (**Figure 5**). It was not consistent for US, microbubbles, and US plus microbubbles to exert similar effects on gene transfer in vitro and in vivo. One possible reason was that in vitro and in vivo cells were in the different physiological conditions. The frequency and intensity of the bioeffects originating from the interaction of ultrasound with tissue might also be responsible for the disparity in US and/or microbubbles-mediated rAAV2 transfection to rat retina in vitro and in vivo.

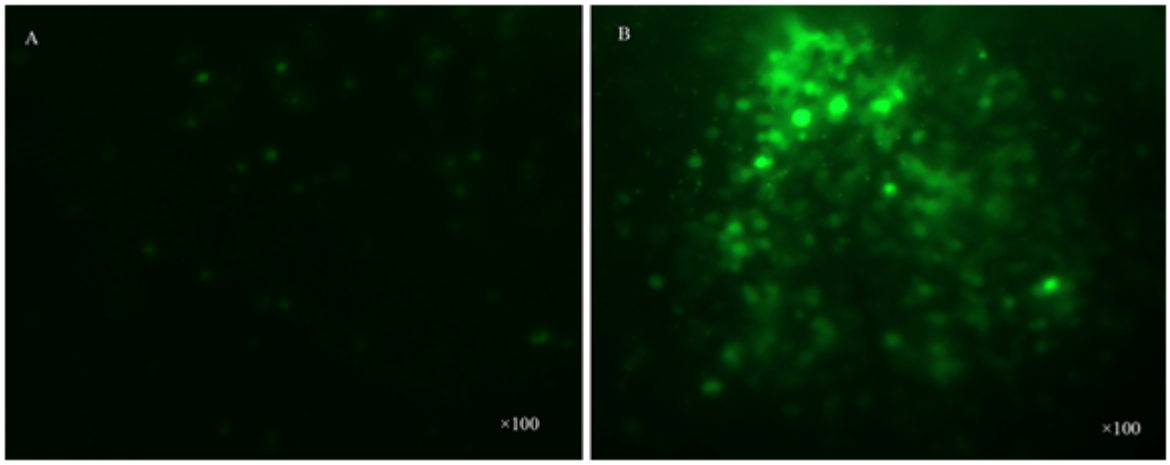

Figure 2. The density of EGFP-positive cells in the retinal tissue-stretched preparation. The number of transfected cells in the group rAAV2-EGFP in combination with UTMD (**B**) was more than that in the group rAAV2-EGFP alone (**A**) on day 35.

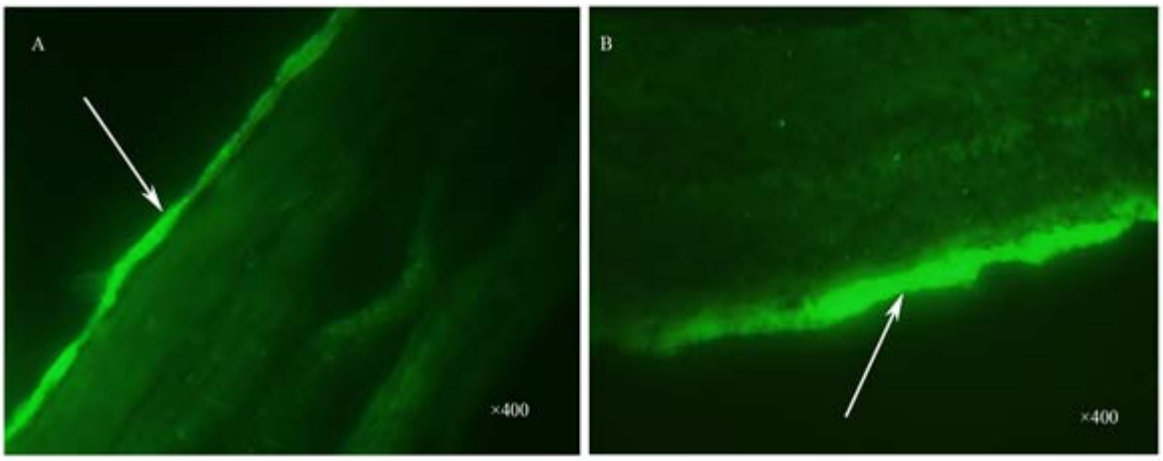

Figure 3. The frozen section of fundus oculi showed that EGFP expression mainly appeared in the layer of RPE cells (**A**) and in the neural retina (**B**).

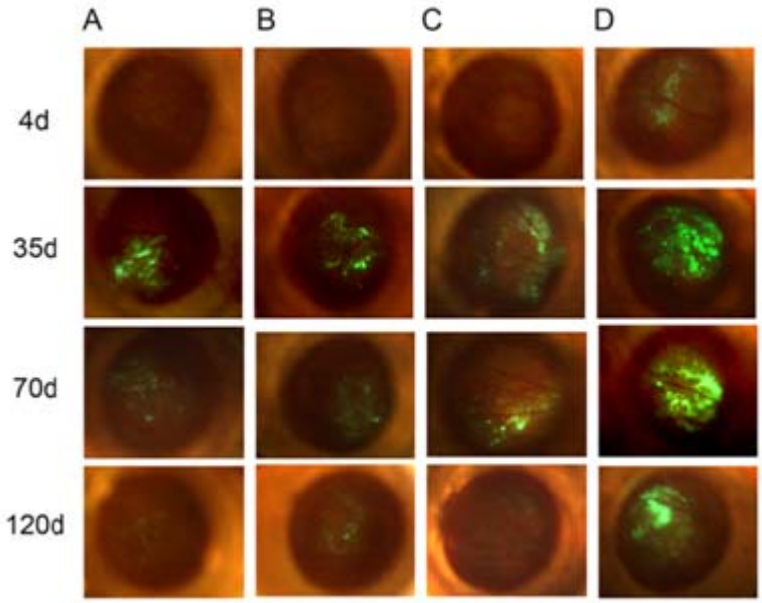

Figure 4. Fluorescence stereoscopic images of EGFP expression at different days (4d, 35d, 70d and 120d) in the ocular fundus of Wistar rats after subretinal injection of (**A**) rAAV2-EGFP alone or in combination with (**B**) microbubbles, (**C**) US or (**D**) US+microbubbles were photographed at different times after subretinal injection (A, B, C and D: 25 x magnification).

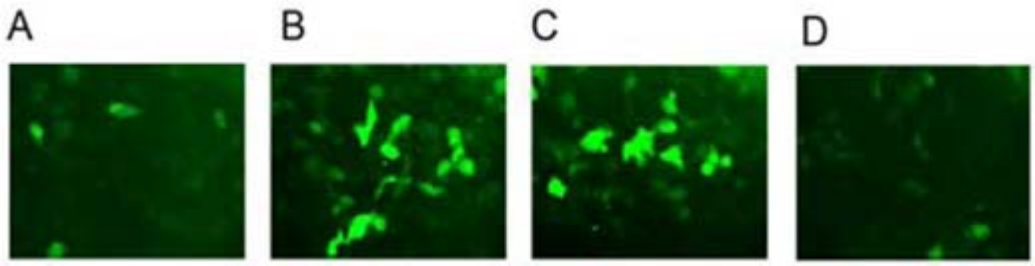

Figure 5. Fluorescence images of EGFP expression in rat PRE cells of four groups: (**A**) rAAV alone, (**B**) rAAV+microbubbles, (**C**) rAAV+US, (**D**) rAAV+US+microbubbles were observed two days after infection. (A, B, C, and D: 400 x magnification).

Enhancement of non-viral gene transfection by UTMD

Although viral gene therapy is very promising, the use of viral vectors has some important disadvantages like the limited size of the expression cassette, difficult large-scale production, and several safety issues like host immunity and oncogenesis [55-58]. Therefore, non-viral gene carriers, based on lipids or polymers, remain interesting for ocular gene therapy. Utilizing nanoparticles as non-viral gene carriers for disease treatment in a wide range of medical research fields has become a popular strategy in recent years. These particles can serve as carriers for drugs, peptides, vaccines, and oligonucleotides. In addition, they have been successfully delivered to multiple targets including cancerous cells and other diseased tissues. Furthermore, Nanoparticles have great potential as a strategy for gene therapy. They can be used to treat genetic defects in vitro and in vivo [59].

Excellent preliminary studies have been undertaken to use polylactic acid, poly (lactide co-glycolide) (PLGA) or chitosan (CS) nanoparticles for gene transfer to the ocular tissues. Bejjani et al. [60] demonstrated that PLGA polyplex NPs were non-toxic and can be used to achieve a gene transfer into RPE cells in vitro and in vivo. In human and bovine RPE cells incubated with GFP loaded nanoparticles, cytoplasmic green fluorescence was observed in

14 1.65% of the cultured cells. In vivo, a preferential red nuclear fluorescent protein (RNFP) expression within the RPE cell layer was detected after intravitreous injection of RNFP plasmid loaded NPs. In a recent research published in the journal of Invest Ophthalmol Vis Sci, de la Fuente et al. [61] presented a novel DNA nanocarrier made of two bioadhesive polysaccharides, hyaluronic acid (HA) and CS. They showed that HA-CS nanoparticles were able to deliver pDNA into both the corneal and the conjunctival cells and transfection efficiency reached 15%, without affecting cell viability. In spite of these successes, there are still barriers to the universal application of nanoparticles as nonviral gene carriers for the treatment of ocular diseases. The biggest problem so far has been the low transfection efficiency seen with some particles and the short duration of gene expression that is typically associated with most non-viral gene therapies.

Some research demonstrates that US or UTMD can effectively deliver the NPs into muscular and tumor tissues [62-65] and interaction of ultrasound with nanoparticles may enhance drug and gene delivery in tumor cells in vivo because it alters properties of tumor vasculature and the cell membrane [65, 66]. This interaction induces non-thermal effects including mechanical membrane stretching due to nanoparticle oscillations, radiation force, and acoustic streaming which all may contribute to the enhancement of drug and gene delivery [65-67].

The neural retina is a multilayer consisting of various cell types. There is some evidence that neural retina as a diffusional barrier limits the nonviral gene transfer to RPE cells [68]. In the Bejjani et al.'s study mentioned above, it was perhaps one of the main causes of low transfection efficiency of PLGA polyplex NPs into RPE cells in vivo by means of intravitreous injection of plasmid-loaded nanoparticles. Peeters et al. [69] indicated that ultrasound energy may be a useful tool to improve the neural retina permeability of the nanoparticles up to 130 nm. This provides an experimental basis for the further enhanced transfection efficiency of nanoparticles into RPE cell by ultrasound combined with microbubbles, due to shock-waves and microjets generated from implosion of these microbubbles during ultrasound exposure, whereas few reports have been published with regard to the enhanced delivery of DNA or siRNA loaded nanoparticles by UTMD in ocular disease.

The blood-retinal barrier opening by UTMD

The blood-retinal barrier is located at two levels, forming an outer barrier in the retinal pigment epithelium and an inner barrier in the endothelial membrane of the retinal vessels [70]. The major challenge for the intravenous administration of drugs and gene is the existence of the inner and outer blood-retinal barrier, which limits the access of drugs and gene to retina. Recently, promising studies [71, 72] indicate that ultrasound exposure combined with microbubble contrast agent can produce the blood-brain barrier opening, and be used to deliver a drug locally or gene to a specific region of interest in the brain. Several avenues of transcapillary passage after ultrasound sonication have been identified, and several hypotheses on the mechanism of the blood-brain barrier disruption with microbubbles. Ultrasound has also been proposed [71]. These results suggested that UTMD could potentially produce the blood- retinal barrier opening. Xu et al. [73] demonstrated that UTMD could effectively deliver EGFP plasmid to the retina compare with gene delivery of naked plasmid after intravenous administration in rats as shown in **Figure 6**. In this study, histology showed no evident tissue damage after UTMD-mediated EGFP transfection. These findings indicated that UTMD could be used to produce the blood-retinal barrier opening leakage substantially without damage to ocular tissue, and served as a promising method for intravenous ocular gene delivery. Further experiments that address the effect of ultrasound and microbubbles upon the various routes of transport across the blood-retinal barrier are necessary.

Safety of ophthalmic application

There have been some concerns regarding the safety of ocular gene transfection mediated by USMT or UTMD. Saito et al. [74] found that ultrasound potently disrupted corneal endothelial cell plasma membrane integrity, resulting in immediate lethal and sublethal cell injury. The degree of lethal cell injury induced by ultrasound scaled with exposure intensity and duration. However, not all cells injured became necrotic, and some survived and appeared to behave normally after exposure if rapid resealing plasma membrane disruption was accomplished. Sonora et al.'s research [40] mentioned above also demonstrated that an exposure time longer than 120 seconds, a duty cycle of 100% or 100% microbubbles significantly damaged the cells, although gene transfer efficiency was high.

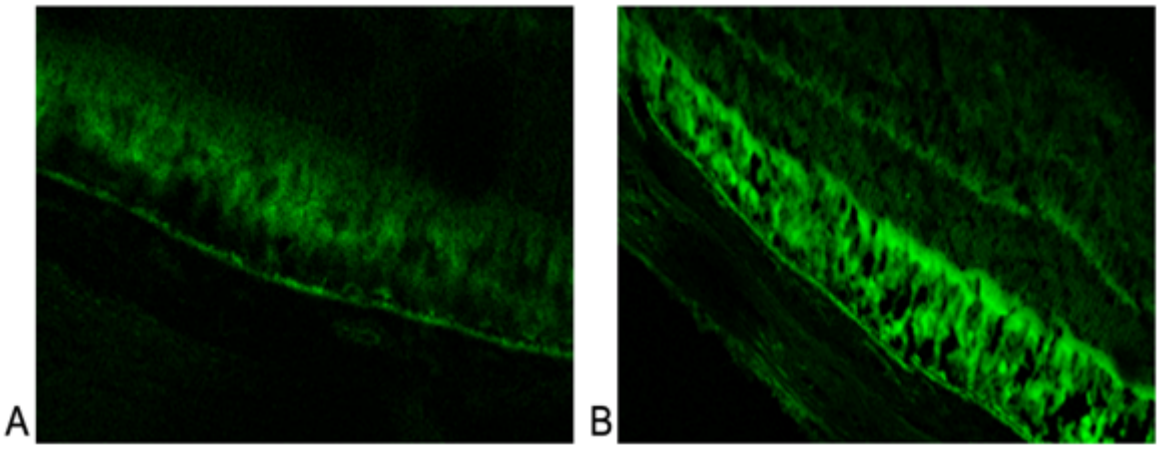

Figure 6. Confocal images of the rats retina showing gene expression of EGFP after different treatments. **A:** Gene delivery of naked plasmid. **B:** UTMD-mediated gene delivery.

It is understandable that high US power can transfer the gene to cells but also induce strong cell damage. The optimal US condition required for efficient gene transfer and less damage must be carefully explored.

Conclusions

UTMD-mediated gene delivery system greatly increases gene transfer to in vitro and in vivo ocular cells. Moreover, US or UTMD, as a promising non-invasive gene transfer method, may enhance viral or non-viral vector-mediated ocular gene delivery. However, there are still several limitations in using therapeutic UCAs in ocular gene transfection at present. The methods to use UCAs for gene delivery are mainly to co-administer or to incubate the microbubbles and the therapeutic together, whereas few reports have been published about the ophthalmic application of the gene-carrying microbubbles that incorporate the gene into the shell or lumen. The bonds between UCAs and DNA are so weak that gene may detach from the surface of the agent before reaching the targeted region after the intravenous administration. Although ultrasound microbubbles are relatively large agents, the amount of drug or gene that can be attached to the bubble surface or incorporated into the internal lipid layer is a concern. The method by which the amount of gene that can be attached to microbubbles can be maximized is also worthy of being studied in future. Additional issue is that present researches only evaluate the tissue structure damage of the retina; a further evaluation of threat to vision is much more important, especially for eventual clinical application.

US with microbubbles have in recent years "come of age" as a potential delivery system for drug and gene transfer. Though several problems remain to be solved, UTMD-mediated gene delivery system is a promising technology for the targeted gene therapy of ocular disease.

Acknowledgments

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