

Comparative analysis of the effects of ultrasound-targeted microbubble destruction on recombinant adeno-associated virus- and plasmid-mediated transgene expression in human retinal pigment epithelium cells

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Received June 19, 2009; Accepted August 19, 2009

DOI: 10.3892/mmr_00000195

Abstract. Ultrasound-targeted microbubble destruction (UTMD) has been utilized to deliver a drug/gene into cells in both *in vitro* and *in vivo* studies. This study was performed to investigate the feasibility of UTMD-enhanced recombinant adeno-associated virus- (rAAV) and plasmid-mediated transfection into the human retinal pigment epithelium (RPE) cell line ARPE-19. Additionally, the transfection efficiency of rAAV and plasmid was compared in order to choose the appropriate gene vector or strategy to be used with UTMD for RPE-based gene modification. rAAV or plasmid encoding an enhanced green fluorescent protein gene was administered to ARPE-19 cells under various UTMD conditions. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Transfection efficiency was determined by fluorescence microscopy and flow cytometry. UTMD significantly enhanced the transfection efficiency of rAAV and plasmid in ARPE-19 cells without adverse effects on cell viability. The transfection efficiency of rAAV was higher than that of plasmid. rAAV is therefore an appropriate gene vector for use with UTMD in retinal gene modification.

Introduction

In their physiological state, retinal pigment epithelium (RPE) cells play an indispensable role in retinal cell biology,

including the phagocytosis of shed rod outer segments and the physiological adhesion of neural retina, the transportation of nutrient, the removal of waste products, the secretion of growth factors and the maintenance of the blood-retinal barrier (1,2). The degeneration of RPE is involved in a wide range of retinal diseases, including retinitis pigmentosa and age-related macular degeneration (1,3). In such pathological states, the deterioration or disorder of RPE function eventually results in loss of vision. Since mature RPE cells do not proliferate *in vivo* and undergo growth only by means of cell enlargement (4), regenerative RPE-based therapies have been employed to repair the function of aberrant RPE. However, the poor viability of transplanted RPE cells limits their therapeutic efficacy. A potential alternative approach with the aim of improving RPE viability and therapeutic efficacy is gene modification.

To date, gene modification has not experienced extensive success. The two major determinants for the success of gene modification are the novel vector used and the feasibility of the gene delivery technique. Multiple viral and non-viral vectors have been investigated. Among the viral vectors, adeno-associated virus (AAV) is an attractive candidate for human gene modification due to its non-pathogenic nature (5) and its ability to infect a wide variety of cells, including ARPE-19. AAV typically provides long-term expression of foreign genes and does not induce inflammation or cytotoxicity (6). However, the transduction of AAV occurs with relatively low transduction efficiency in some tissues, which limits its therapeutic effectiveness. Among the commonly used non-viral vectors are naked plasmids. These are relative safe, have the capacity to transfer large genes, and are site-specific, non-inflammatory and non-toxic (7). Although naked plasmids are free of virus-associated adverse effects, their transduction efficiency is also low and their transgene expression is relatively poor.

Ultrasound-targeted microbubble destruction (UTMD) is a potential site-specific gene transfer modality that has been gradually developed over the past two decades. Studies have confirmed that UTMD can improve the cellular uptake of polynucleotides (8,9), proteins (10) and small-molecule drugs (11). Thus, it is regarded as a promising gene delivery technique for gene modification.

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Key words: ultrasound-targeted microbubble destruction, gene expression, recombinant adeno-associated virus 2, plasmid, ARPE-19 cells

In this study, we hypothesized that the intracellular delivery of rAAV- and plasmid-mediated transgene into human RPE cells by UTMD could lead to improvements in cellular function and viability. Consequently, the feasibility of UTMD-enhanced rAAV and plasmid transfection into ARPE-19 cells was examined, and their transfection efficiency was compared in order to choose an appropriate gene vector or strategy for use with UTMD in RPE-based gene modification.

Materials and methods

Recombinant adeno-associated virus. The rAAV2-EGFP was purchased from Vector Gene Technology Company Ltd. (Beijing, P.R. China) and expressed the transgene under the control of the cytomegalovirus promoter.

Plasmid preparation. An expression vector for the EGFP gene, pEGFP-N1 (4.7 kb), was provided by the Experimental Research Center (Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, P.R. China). Plasmid DNA was purified from a culture of *Escherichia coli* DH5a using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. The purity of the plasmid DNA was determined by measuring absorption at a 260-nm wavelength (A260) using spectrophotometry. The A260:A280 ratio of pEGFP-N1 was between 1.8 and 1.9, indicating that the purified DNA was free of proteins and RNA. All experiments were performed using the same preparation of plasmid DNA.

Microbubble contrast agents. SonoVue[®] microbubble contrast agent (Bracco, Milan, Italy) was reconstituted in saline solution according to the manufacturer's protocol, and yielded a preparation containing 2.5×10^8 microbubbles (MBs)/ml by inversion/agitation of the unit. This microbubble contrast agent is a composition of a core of sulfur hexafluoride gas and an envelope of phospholipids. The average diameter of the MBs was 2.5-6.0 μm .

Cell culture. The human RPE cell line ARPE-19 (ATCC, Rockville, MD, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C, 5% CO₂/95% air.

Cell viability. The cell viability of ARPE-19 under the influence of UTMD was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. A therapeutic ultrasound machine (Topteam161, Chattanooga, TN, USA) and a 1-cm² probe were applied in this experiment. The ARPE-19 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and grown in a humidified incubator at 37°C, 5% CO₂/95% air for 24 h. When the cells reached 70-80% confluence, the medium in each well was drawn off and replaced with 100 μl fresh DMEM for the the control group, or with a 100- μl mixed solution of MBs and fresh DMEM (at MB concentrations of 20, 50 and 100%) for use in the UTMD group. The frequency and pulse recurrent frequency (PRF) of the ultrasound (US) were set at 1 MHz and 50 Hz, respectively. Additional parameters of US irradiation were: intensity, 1, 2 and 3 W/cm²; duration, 1, 2 and 4 min; duty cycle, 50%. For insonation, the probe

was placed at the bottom of the plates with a small amount of coupling medium on its surface. A home-made plastic disc with a hole the same size as the wells in its middle was placed between the probe and the bottom of the plates to ensure the same thickness of coupling medium between the probe and the plates, and to prevent ultrasonic radiation from affecting consecutive wells. After US exposure, each well was supplemented with 100 μl of DMEM, then the ARPE-19 cells were cultured for an additional 24 h. Subsequently, 20 μl of 5 mg/ml MTT solution in PBS was added to each well. After a 4-h incubation at 37°C, 5% CO₂/95% air, the supernatant was removed and the formazan product was washed out by treatment with 150 μl dimethyl sulfoxide. The absorbance of each well was measured in an enzyme-linked immunosorbent assay reader at 570 nm. Untreated cells served as the reference and were taken as 100% viability. The percentage of cell viability was calculated using the equation: cytoactive (%) x optical density (OD) of the treatment group/OD of the control group x100%.

Experimental grouping. The ARPE-19 cells were placed in every other well of 24-well plates at a density of 2×10^5 cells/well and incubated in 500 μl DMEM with 10% FBS for 24 h prior to transfection. The cells were then respectively co-cultured with rAAV2-EGFP alone at a multiplicity of transfection (MOI) of 1×10^3 vector genomes per cell (vg/cell) (group R alone) or with pEGFP-N1 alone at a concentration of 10 μg /well (group P alone), and in combination with MBs (group R+MBs and group P+MBs), US (group R+US and group P+US) or UTMD (group R+US+MBs and group P+US+MBs).

Ultrasound exposure. The above-mentioned therapeutic ultrasound machine and a 2-cm² probe were applied in this experiment. The parameters of UTMD were in accordance with those of the cell viability test (1 and 2 W/cm² intensity, 1 and 2 min duration, and 50% duty cycle). MB concentrations of 13.3, 20, 26.7, 33.3, 40, 50 and 100% were selected. Prior to US exposure, the DMEM in the 24-well plates was drawn out and a small amount of fresh serum-free DMEM was added to the medium, ensuring that the volume per well was 150 μl . rAAV2-EGFP and pEGFP-N1 were diluted by DMEM and mixed with isovolumic MBs, then left standing for 5 min. The final solution was added to the plates and exposed to US as described above. Each of the 24-well plates was supplemented with medium to a final volume of 500 μl at 2 h post-transduction, and was replaced with fresh DMEM with 10% FBS 12 h after transfection.

Gene transfection efficiency. Following gene transfection, the ARPE-19 cells of the rAAV-EGFP and pEGFP-N1 groups were incubated in DMEM with 10% FBS for 48 h. EGFP expression was observed and photographed using inverted fluorescence microscopy (Zeiss Axiovert S100, Jena, Germany). EGFP expression of the transduced ARPE-19 cells was quantitatively examined by FCM analysis (EPICS XL, Beckman Coulter Co., Miami, FL, USA).

Statistical analysis. Data were expressed as the means and standard deviation (mean \pm SD). Analysis of variance (ANOVA) was used to test for significance by multiple comparison. Differences were considered significant at $p < 0.05$. SPSS version 13 (SPSS, Chicago, IL, USA) was used for all statistical analysis.

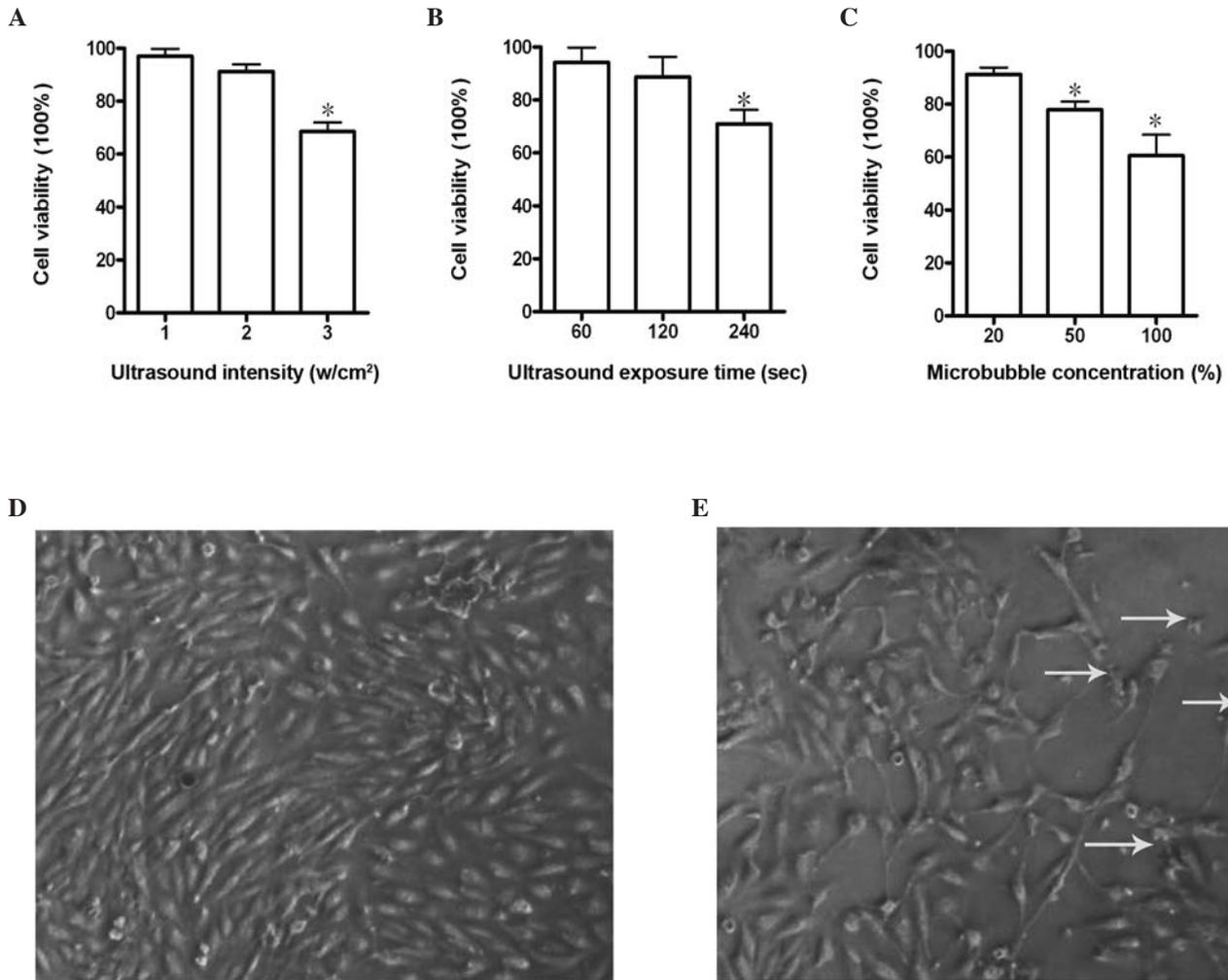


Figure 1. Cell viability of ARPE-19 cells assessed by MTT assay at various (A) ultrasound intensities, (B) exposure durations and (C) microbubble (MB) concentrations. The data indicated that an ultrasound intensity of 1 and 2 W/cm², duration of 60 and 120 sec, MB concentration of 20%, and 50% duty cycle were the optimal conditions for the mediation of gene transfection to ARPE-19 cells (*p<0.05). (D) Phase-contrast image of ARPE-19 cells immediately after ultrasound-targeted microbubble destruction (UTMD) (1 and 2 W/cm², 60 and 120 sec, 20% MBs, 50% duty cycle). No cell damage was apparent. (E) Phase-contrast image of ARPE-19 cells immediately after UTMD (3 W/cm², 60 sec, 100% MBs, 50% duty cycle). Cell damage was apparent in many cells (arrows). (D and E, magnification x100). Data are presented as the means ± SD of three independent experiments.

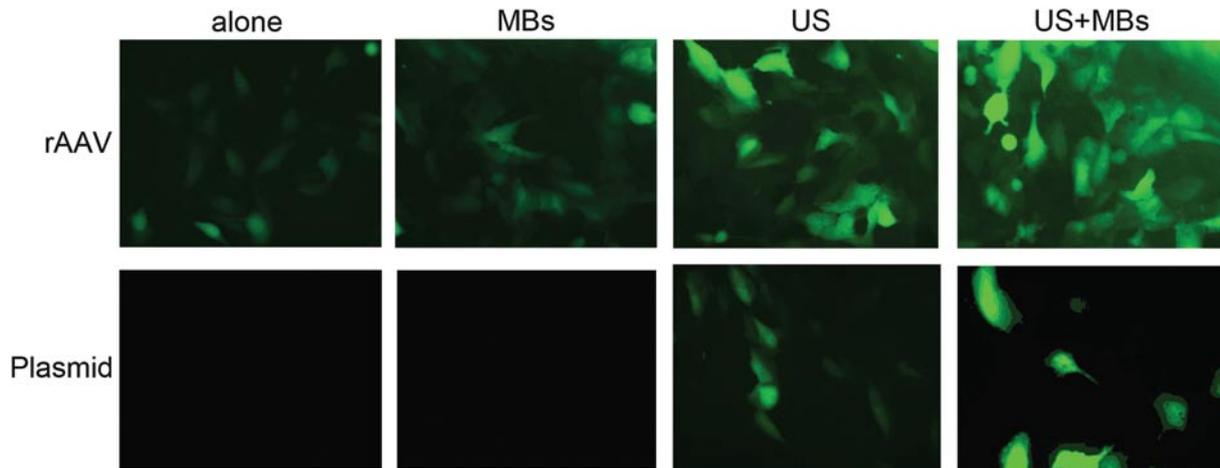
Results

Effect of ultrasound-targeted microbubble destruction on cell viability. The effects of UTMD on the viability of ARPE-19 cells are shown in Fig. 1. When the duty cycle was set at 50% with a 20% MB concentration, the increase in US intensity (from 1 to 3 W/cm²) and duration (from 60 to 240 sec) resulted in decreased cell viability. Cell viability was lowest in the 3 W/cm² group (74.58±10.46%) and the 4 min group (72.93±8.33%). No significant difference was found between the 1 W/cm² and 2 W/cm² groups (97.07±10.70 vs. 91.29±2.62%), nor between the 1 min and the 2 min groups (91.29±2.62 vs. 88.75±7.59%) (Fig. 1A and B). With a 2 W/cm² intensity and 60-sec duration, the increase in MB concentration (from 20 to 100%) resulted in an increase in cell mortality (Fig. 1E). Cell viability was lowest in the 100% MB concentration group (60.58±7.91%), and there was significant difference between the 20% and the 50% MB concentration groups (91.29±2.62 vs. 77.97±2.96%) (Fig. 1C). Thus, an MB concentration of 20%, US intensity of

1 and 2 W/cm², duration of 60 and 120 sec and 50% duty cycle were determined to be the optimal conditions for the mediation of gene transfection to ARPE-19 cells (Fig. 1D).

Gene transfer by microbubbles, ultrasound and ultrasound-targeted microbubble destruction. No improvement in transgene expression was observed in the R+MB and P+MB groups. Compared to the R and P alone groups, the R+US (21.47±0.53 vs. 18.23±0.65%) and P+US (1.06±0.13% vs. 0) groups showed a weak but non-significant tendency to improve transgene expression. The ratio of EGFP-positive cells was significantly higher in the R+US+MB and P+US+MB groups (1.5 to 2-fold and 10 to 15-fold, respectively) compared to the US alone groups under any of the following experimental conditions: 1 W/cm², 60 sec, 50% duty cycle, 20% MB concentration; 1 W/cm², 120 sec, 50% duty cycle, 20% MB concentration; 2 W/cm², 60 sec, 50% duty cycle, 20% MB concentration; 2 W/cm², 120 sec, 50% duty cycle, 20% MB concentration (Fig. 2A and B).

A



B

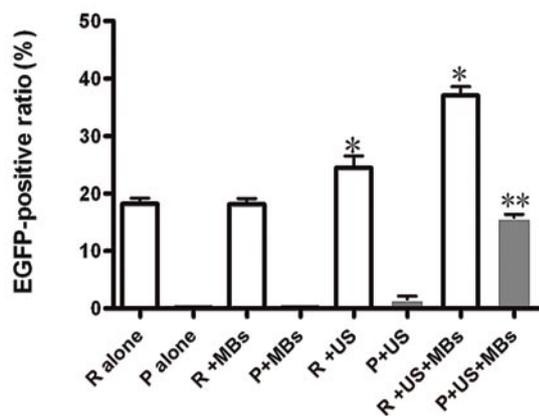


Figure 2. Transfection efficiency of recombinant adeno-associated virus (rAAV) and plasmid in ARPE-19 cells alone, with microbubbles (MBs), with ultrasound, and with ultrasound-targeted microbubble destruction (UTMD). (A) EGFP expression in ARPE-19 cells observed 48 h after transfection with rAAV2-EGFP and pEGFP-N1 (magnification $\times 400$). (B) EGFP-positive rate of ARPE-19 cells analyzed by flow cytometry 48 h after transfection with rAAV2-EGFP and pEGFP-N1 (** $p < 0.01$ and * $p < 0.05$, respectively). In all the experiments, the ARPE-19 cells were transfected with rAAV2-EGFP at a concentration of 1×10^3 vg/cell and with pEGFP-N1 at $10 \mu\text{g}/\text{well}$. Data are presented as the means \pm SD of three independent experiments.

Optimization of ultrasound-targeted microbubble destruction parameters in the recombinant adeno-associated virus- and plasmid-mediated gene transfection. To identify the optimal conditions for transferring genes by UTMD in the R+US+MB and P+US+MB groups, the following three parameters were examined:

Microbubble concentration. Four concentrations of MBs (13.3, 20, 26.7 and 33.3%) in the R+US+MB group were examined under conditions of $1 \text{ W}/\text{cm}^2$ US intensity, 60-sec duration and 50% duty cycle. Three concentrations of MBs, (20, 50 and 100%) in the P+US+MB group were examined under the conditions of $2 \text{ W}/\text{cm}^2$ US intensity, 60 sec-duration and 50% duty cycle. In both groups, the EGFP-positive ratio was highest in cells treated by US with an MB concentration of 20% (Fig. 3A and B). Thus, a 20% MB concentration was preferable.

Ultrasound intensity. US intensities of 1, 2 and $3 \text{ W}/\text{cm}^2$ were examined under conditions of 50% duty cycle, 60-sec duration and 20% MB concentration in the R+US+MB and P+US+MB groups. The EGFP-positive ratio was smaller at US intensities of $3 \text{ W}/\text{cm}^2$ ($32.92 \pm 0.97\%$) compared to 1 and $2 \text{ W}/\text{cm}^2$. No significant difference between the EGFP-positive ratio under 1 and the $2 \text{ W}/\text{cm}^2$ conditions (37.05 ± 0.55 vs. $35.74 \pm 0.42\%$, respectively) was found in the R+US+MB group

(Fig. 3C). However, in the P+US+MB group, an increase in US intensity resulted in an increase in the EGFP-positive ratio (5.77 ± 0.67 , 15.81 ± 1.70 and $20.86 \pm 2.63\%$ for 1, 2 and $3 \text{ W}/\text{cm}^2$, respectively) (Fig. 3D). Taking into consideration the effect of UTMD on cell viability, US intensities of $1 \text{ W}/\text{cm}^2$ in group R+US+MB and $2 \text{ W}/\text{cm}^2$ in group P+US+MBs were selected.

Exposure duration. US exposure durations of 60 and 120 sec were examined under conditions of $1 \text{ W}/\text{cm}^2$, 50% duty cycle and 20% MB concentration in the R+US+MB group and under conditions of $2 \text{ W}/\text{cm}^2$, 50% duty cycle and 20% MB concentration in the P+US+MB group. The EGFP-positive ratio was significantly higher with a 60-sec duration than with a 120-sec duration (37.05 ± 0.55 vs. $28.13 \pm 0.67\%$ in group R+US+MBs and 15.81 ± 1.70 vs. $11.31 \pm 1.99\%$ in group P+US+MBs) (Fig. 3E and F). Consequently, an exposure duration of 60 sec was determined to be appropriate in both groups.

Discussion

To date, the viral gene vector rAAV and non-viral gene vector plasmids have been extensively employed in gene modification for curing certain inherited or acquired retinal disorders. AAV is a single-stranded DNA-containing defective parvovirus.

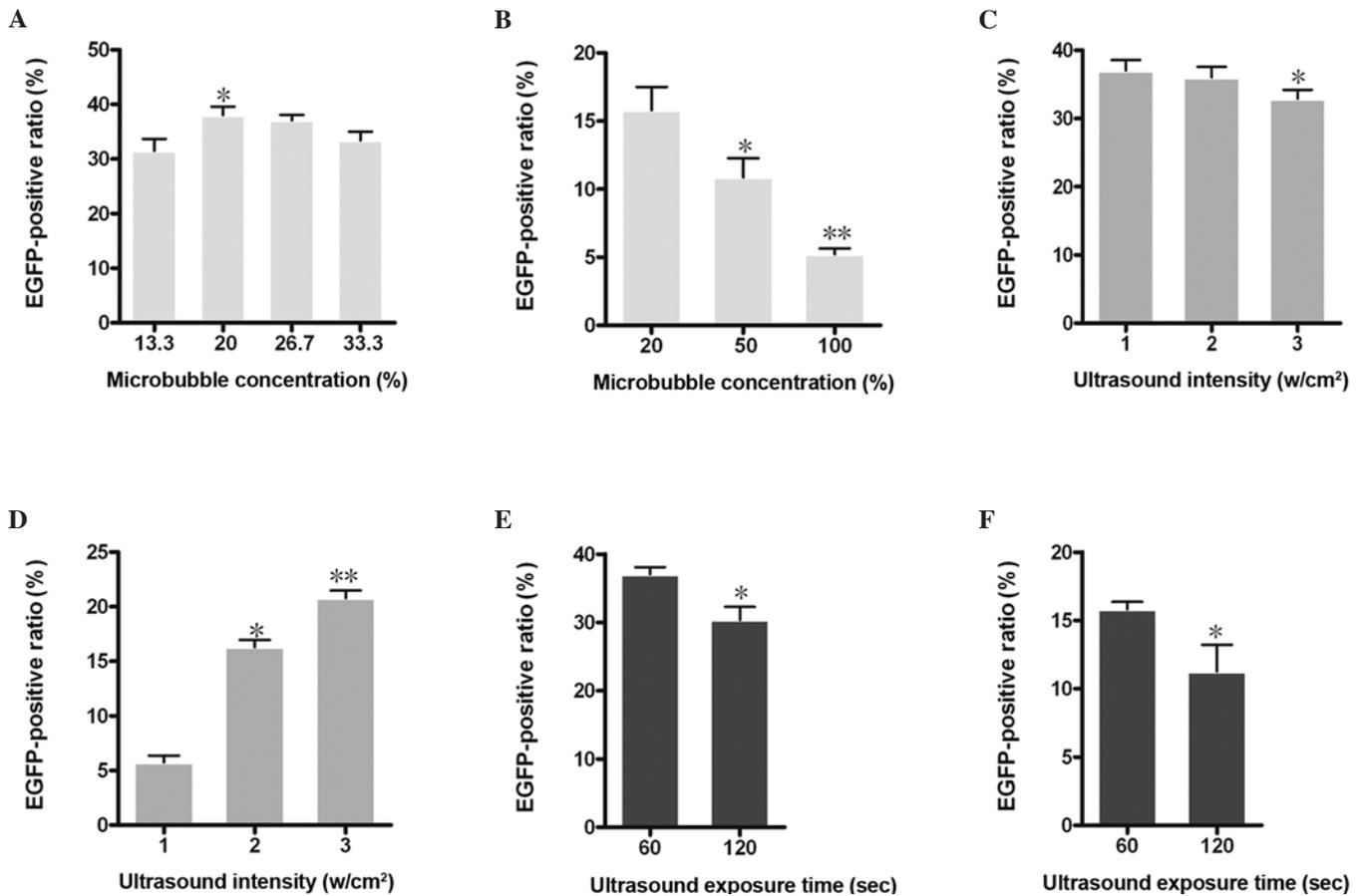


Figure 3. Gene transfer by ultrasound-targeted microbubble destruction (UTMD) under various conditions. Flow cytometry was used to examine the EGFP-positive ratio 48 h after transfection with: various concentrations of microbubbles and (A) rAAV2-EGFP and (B) pEGFP-N1; various ultrasound intensities and (C) rAAV2-EGFP and (D) pEGFP-N1; various ultrasound exposure durations and (E) rAAV2-EGFP and (F) pEGFP-N1 (** $p < 0.01$ and * $p < 0.05$, respectively). In all the experiments, the ARPE-19 cells were transfected with rAAV2-EGFP at a concentration of 1×10^3 vg/cell and with pEGFP-N1 at $10 \mu\text{g}/\text{well}$. Data are presented as the means \pm SD of three independent experiments.

Lack of efficiency in genome conversion is an obstacle to gene transfection, limiting its therapeutic effects. Several studies have found that a variety of biological, chemical and physical events, such as co-transfection of target cells with a helper virus or adenovirus (3), treatment with hydroxyurea or etoposide (12), or ultraviolet (13,14) or γ -ray irradiation (15), can greatly enhance the efficiency of AAV-mediated transduction, presumably by catalyzing genomic conversion. However, these techniques may have marked adverse effects, such as innate host antiviral immune responses, cell toxicity and tissue damage. Gene delivery with plasmid vectors is highly inefficient unless the DNA is associated with other molecules and/or physical energy is applied to aid cell entry, for example through electroporation, gene gun or hydrodynamic pressure (16). These techniques often cause substantial tissue damage, making them inappropriate for clinical application.

US is widely used for clinical imaging, and its safety has been reliably established. Recent studies have demonstrated that UTMD can be used to enhance adenoviral or plasmid transgene transduction efficiency, reducing their concentrations and directing tissue expression of adenoviral or plasmid transgenes to a specific organ (17,18). However, little research has been reported regarding UTMD enhanced rAAV- or plasmid-mediated gene transfection into RPE cells. The detailed

mechanisms of UTMD involvement in gene transfection have yet to be fully explained. It is thought that the bioeffects of UTMD, such as cavitation, thermal effect, radiation force and chemical effect combined, may result in permeability changes to the cell membrane, therefore leading to the increased uptake of transgenes (10,19,20). In addition to permeabilization of the cell membrane, locally and momentarily-generated energy released by the collapse of MBs acts as a motivational force, overcoming the physical obstacle of the plasma membrane. Thus, foreign genes of interest can pass into the cell, and the intracellular shock wave can encourage the vector DNA or RNA to exit the endosome and enter the nucleus.

The results presented here suggest that higher rAAV and plasmid transfection efficiency can be obtained in ARPE-19 cells by UTMD, though not by US or MBs alone. To establish the optimal conditions for UTMD in the rAAV-EGFP and pEGFP-N1 groups, the effects of UTMD on ARPE-19 cell viability were initially assessed. Three UTMD variables, MB concentration, US intensity and US exposure duration, were sequentially examined, and the optimal conditions that could be safely applied for gene transfection to ARPE-19 cells were obtained. Taking into consideration gene transfection efficiency and cell viability, the optimal UTMD conditions were $1 \text{ W}/\text{cm}^2$ US intensity, 60-sec duration, 50% duty cycle and 20% MB

concentration in the rAAV-EGFP group, and 2 W/cm² US intensity, 60-sec duration, 50% duty cycle and 20% MB concentration in the pEGFP-N1 group.

Although the transfection efficiency of plasmid enhanced by UTMD was markedly elevated compared to that of plasmid alone (from 0 to >10%), it was still far below the threshold needed for efficient gene modification, and was not further elevated by increasing the concentration of plasmid. For example, when the concentration of plasmid was increased from 6 to 10 µg/well, transfection efficiency only increased from 12.58±1.75 to 15.81±1.70% (data not shown). In contrast, the transfection efficiency of rAAV enhanced by UTMD was just below or at the level required for gene therapy. Moreover, increasing the concentration of rAAV improved therapeutic efficacy. For example, the transfection efficiency of rAAV treated by UTMD increased from 37.05±0.55% to 53.23±1.09% when the MOI of rAAV was increased from 1×10³ vg/cell to 1×10⁴ vg/cell (data not shown). Therefore, rAAV may be an appropriate gene vector for use with UTMD treatment in retinal gene modification.

The difference in the effect of UTMD on the transfection efficiency of rAAV-EGFP and pEGFP-N1 in ARPE-19 cells is primarily due to the different transfection mechanisms of AAV and plasmid. AAV initiatively transfers into ARPE-19 cells by adhesion to their surface receptors, while plasmid is only transferred into ARPE-19 cells passively by other materials or by energy. Additionally, AAV and plasmid have different biological characteristics, such as their molecular weight, distribution of microstructures and trait of CHARGE, leading to different modalities and results of interaction between ARPE-19 cells and rAAV-EGFP or pEGFP-N1, and thus different transfection efficiency. The subtle mechanisms underlying these phenomena are complicated and require further elucidation.

In summary, our *in vitro* findings demonstrate that UTMD is capable of significantly enhancing the transfection efficiency of rAAV and plasmid in ARPE-19 cells without adverse effects on cell viability. In ARPE-19 cells, the optimal UTMD conditions for mediating rAAV and plasmid transfection are, respectively, 1 W/cm² US intensity, 60-sec duration, 50% duty cycle and 20% MB concentration, and 2 W/cm² US intensity, 60-sec duration, 50% duty cycle and 20% MB concentration. In retinal gene modification, rAAV may be an appropriate gene vector for use with UTMD.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant no. 30772369). The authors gratefully acknowledge the entire staff at the Experimental Research Center of Shanghai Jiaotong University Affiliated First People's Hospital for technical assistance and helpful discussion.

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