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Original Article

Acoustic waves improves retroviral transduction in human retinal stem cells

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Abstract

Backgrounds: The plasticity of retinal stem cells (RSCs), a type of cells that can differentiate into neuron cells and photoreceptor cells, endows them with potential therapeutic properties that can be applied to regenerative medicine. Gene modification of these stem cells before trans-differentiation and transplantation enhances their survival and increases their therapeutic function. The different ways to effectively deliver gene into RSCs are still discussed. This study aimed to use the acoustic waves to improve the efficacy of gene delivery for RSCs.
Methods: RSCs were obtained from non-fetal human ocular pigmented ciliary margin tissues. The enhanced green fluorescent protein-encoded murine stem cell retroviruses (MSCV) were prepared and used to infect RSCs. Glass chambers containing RSCs, retroviruses, and various concentrations of polybrene (0, 0.8, 2, 4 and 8 μg/mL) were exposed under 20 or 25 Vp-p ultrasonic standing wave fields (USWF) for 5 min. The percentage of green fluorescent protein positive cells in each sample was calculated and compared to test the efficacy of gene transduction.
Results: Our results showed that the efficiency of gene transduction by MSCV infection was enhanced following the concentration of polybrene and the energy of USWF. The percentage of green fluorescent protein positive cells was significantly higher in chambers that contained 8 μg/mL of polybrene and was exposed to 20Vp-p of USWF for 5 min. In addition, the percentage increased in chambers contained 2, 4 and 8 μg/mL of polybrene when they were exposed to 25Vp-p of USWF. Comparing to those did not treated with ultrasound, the efficiency of retroviral transduction to RSCs increased 4-fold after exposed to USWF for 5 min.
Conclusion: We demonstrated the ability of ultrasound standing waves to improve retroviral transduction into RSCs. We believe that this may be applied to the experimental designs of future studies and may have possible therapeutic uses.

Keywords: Gene transfection; Photoreceptor; Retinal stem cells; Retrovirus; Ultrasound standing waves

1. Introduction

The human retina is composed of ten layers of different cell types. Damage or loss of any of them would result in permanent visual disturbance or blindness.[1,2] Various treatments have been tried to restore vision loss caused by cells damaged in retinal diseases, but the results have been unsatisfactory.[3] Retinal stem cells (RSCs) have the capacity of self-renewal

Conflict of Interest: The authors have no proprietary or commercial interests in any concept or product discussed in this article.

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and can differentiate into various specific retinal cells. Positive results from stem cell-based therapies have been demonstrated for treating some retinal diseases. MacLaren et al. have taken stem cells at later developmental stages and transplanted them into adult mice with photoreceptor loss, thereby demonstrating that there is a particular time window of development for transplant success in restoring sight to the blind mice.[4] This proof-of-principle breakthrough, transplantation of RSCs that results in photoreceptor cells forming new connections to the mature retina, offers promise for the regenerative treatment of retinal disease.

Transfection of specific genes into stem cells would trigger their transdifferentiation and enhance expression of various trophic factors and crucial proteins from these stem cells. It is an effective way to generate stem cells and improve the suitability for transplant. Gene transduction can be performed either by viral or non-viral approaches. Unfortunately, eukaryotic cells have evolved high barriers to the entry of foreign DNA. Methods to improve the efficacy of gene transduction before stem cell therapy has been widely discussed recently.

Ultrasound-assisted gene transfer (UAGT) has been known as a basis of "sonoporation," in which microbubbles are applied to the cell membrane, destroying areas of the transmembrane to allow macromolecules to enter the cell.[5] There is increasing evidence that exposure of eukaryotic cells to ultrasound of relatively modest intensity, within the range emitted by diagnostic transducers in combination with other viral techniques, can enhance transgenic expression by up to several orders of magnitude over naked DNA alone. In combination with the clinical safety profile of ultrasound, it is suggested that ultrasound-assisted gene delivery has great promise as a novel approach to improve the efficiency of retroviral gene delivery. However, the cavitation effects that occur in a liquid medium with UAGT can cause cell structure to be destroyed as well.

To solve this dilemma, we tried using ultrasonic standing wave fields (USWF) for gene transfer into RGCs in this study. RSCs were exposed to USWF at different exposure times, voltages, and concentration of polybrene. We utilized murine stem cell virus (MSCV) as the gene carrier and examined the effect of USWF on the retroviral gene delivery system.

2. Methods

2.1. Human RSCs

This study was approved by the institutional review board of the Taipei City Hospital. RSCs were collected from non-fetal human sources, with the procedure briefly being described in the following. Strips of the pigmented ciliary margin (PCM) tissues of non-fetal human eyes were separately incubated in Hank's Balanced Salt Solution (HBSS) containing 0.05% trysin for 10 min at 37 °C and were mechanically dissociated with Pasteur pipettes in a Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) medium (Gibcol) at 1:1 ratio. Dissociated cells then were centrifuged at 150 g for 5 min, the enzyme solution was removed, and viable cells were counted by trypan blue exclusion and plated as 5000 cells/200 μL per well in 96-well plates (Corning, Acton, MA) replaced with serum-free culture media composed of DMEM/F-12 medium (Gibcol) with insulin (25 μg/mL; Sigma), transferrin (100 μg/mL; Sigma), progesterone (20 nM; Sigma), putrescine (60 μM; Sigma), sodium selenite (30 nM; Sigma), and human recombinant EGF 20 ng/mL and bFGF 20 ng/mL (R&D Systems, Minneapolis, MN).

The number of primary spheres generated in each well was assessed seven days after plating. The primary suspended spheres were mechanically dissociated into single cells by trituration and an aliquot was counted to determine the total number of cells. For secondary cultures, 500 cells/200 μL per well were plated in each well in 96-well plates using the same culture conditions. The number of secondary spheres in each well in each culture condition was scored seven days after plating. The primary and secondary isolated spheres were plated on poly-L-ornithine-coated (15 μg/mL) glass coverslips in individual wells of 24-well plates (1.0 mL/well) in DMEM/F-12 medium containing 2% fetal calf serum (FCS; Gibcol) and without epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Coverslips were processed eight days later and fixed with 4% paraformaldehyde for 20 min.[6]

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

For RT-PCR, the total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) as described previously.[7] In brief, the first strand of cDNA was synthesized by extending the oligo-dT primer with 25 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Promega, Madison, WI, USA) in a mixture containing 1 μg total RNA, 6 mM $MgCl_2$ and 2 mM Deoxynucleotide (dNTP) at 37 °C for 1 h. PCR of the cDNA was performed in a final volume of 50 μl containing 0.2 mM dNTP, 2 mM $MgCl_2$ 20 pmole of opsin primer and 5 μl first strand cDNA. Amplification of β-actin served as the internal-control for sample loading. The primers for RT-PCR were as follows: THf: TTCTGGA-ACGGTACTGTGGCTA (nt 1140-1161, GenBank accession no. L22651), THr: TGGGAGAACTGGGCAAATGT (nt 1397-1416); 5-HTf: AAGAGGGAAGGAGATGGTGGAT (nt 79-100, GenBank accession no. U31884), 5-HTr: AGCC-CAGGAGAAGCCAATGC (nt 354-373); Bcl-2f: TGTCA-CAGAGGGGCTACGAG (nt 302-321, GenBank accession no. L14680), Bcl-2r: GAGCGATGTTGTCCACCAGG (nt 729-748). FGFR1(f), 5'-ATGGCACCCGAGGCAT TATT-3' (nt 2725-2744, GenBank accession no. NM_000604), FGFR1(r), 5′-GGCT CATGAGAGAAGACGGAAT-3' (nt 3125-3104). Reactions were prepared in duplicate and heated to 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 min, annealing at 55 °C for 5 min, and extension at 72 °C for 20 min.

2.3. Cell viability assay for RSCs

RSCs were seeded on 24-well plates and methyl-thiazol tetrazolium assay (MTT assay; Sigma–Aldrich Co.) was performed for cell viability. RSCs were incubated with 0.25 mg/mL MTT for 4 h at 37 °C, and the reaction was terminated by the addition of 100% isopropanol. The amount of MTT formazan product was determined via a microplate reader and the absorbance was measured at 560 nm (SpectraMax 250, Molecular Devices, Sunnyvale, CA, USA).

2.4. Preparation of Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped murine stem cell retrovirus (MSCV).

The cDNA plasmids of mi–green fluorescent protein (miGFP), pMD, VSV-G of MSCV (MSCV; a gift from Shih CC and Yee JK; City of Hope, Duarte, CA, USA) have been described previously. To generate GFP retroviral supernatants, 293 cells were transiently transfected by calcium phosphate-mediated coprecipitation with 5 μg of the plasmids. The cells were fed at 24 h post infection, and the retroviral supernatant was collected at 48 h. The cells continued to produce high-titer retrovirus for another two days, and that supernatant was collected. The supernatant was filtered through a 0.45 μm pore-sized filter and stored at −80 °C for later use.

2.5. Setup of acoustic apparatus

An USWF was generated at 1 MHz by applying a continuous sinusoidal (ranging from 20 to 30 Vp-p) amplifier output signal to a piezoelectric transducer made of a 25-mm diameter and 1-mm thickness lead zirconate titanate disc (model PZT5800; Channel Industries Inc., Santa Barbara, CA, USA) bonded on a plastic platform to bring retroviruses and cells into close contact. The oscillation signal was generated from a 16-MHz function generator card (model NI PCI-5401; National Instruments Corp., Austin, TX, USA), which was driven by a computer, and it was further transferred through an amplifier (model 7500; Krohn-Hite Corp., Brockton, MA, USA), and an impedance matching transformer (model MT-56; Krohn-Hite Corp., Brockton, MA, USA). The acoustic chamber was a transparent cylindrical tube made of glass with dimensions of 13-mm inner diameter, 38-mm height, and 0.5-mm thickness. Before placing in the acoustic chamber, the transducer was smeared with glycerol as the coupling agent on the surface. After the tube was filled with the medium containing cells and retroviruses, the chamber was capped with a glass coverslip to provide a smooth acoustic reflector for the generation of USWF.[8]

2.6. Effect of polybrene concentration under USWF exposure

A total of 1×10^6 RSCs were suspended in 75 mL of retrovirus-containing media. Five milliliters of the cell suspension were distributed equally into 15 glass chambers. To examine the effect of cationic polymers on facilitating transduction rate via neutralizing electrostatic repulsion force between cells and retroviruses, five glass chambers had polybrene added separately to obtain concentrations of 0, 0.8, 2, 4, and 8 μg/mL and were operated under 20 Vp-p USWF exposure for 5 min. Another five glass chambers had polybrene added in the same manner described above, except having no USWF treatment. The control group was the remaining five chambers operating without MSCV but under USWF exposure. Another duplicate set of experiments was done with 25 Vp-p USWF exposure.

2.7. Statistical analysis

The results of the experimental data were done in triplicate and expressed as mean ± standard error, and an unpaired Student *t*-test was used to evaluate the significant difference. A *p*-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Isolation of RSCs from human donors

For in vitro culture of RSCs, we harvested freshly isolated RSCs from the eyes of three postmortem donors (8 months-old, 37 years-old, and 68 years-old). The harvesting and culture of RSCs was done as previously described. The dissected PCM tissues were trypsinized and dissociated by a mesh procedure and cultured in EGF serum-free medium. A total of 100,000 cells from donors were cloned and cultured in a 24-well culture well. Over four consecutive weeks, these samples were observed and the formation of retinal pigmented and non-pigmented neurospheres were counted (Fig. 1).

3.2. Temporal progress of cell aggregation in USWF

The apparatus to generate USWF for concentrating cells and trapping retroviruses onto the nodal planes within a cylindrical chamber is shown in Fig. 2. A series of photographic images illustrate the formation and evolution of cell bands under USWF exposure at 1 MHz and 25 Vp-p (peak to peak). The evolution of cell bands in USWF chambers was similar to the one described in the previous report.[8]

3.3. Different concentrations of polybrene for RSCs survival

In different concentrations of polybrene, survival of RSCs was tested by measuring cell viability by MTT assay 24 h after inception, and the experimental results are shown in Fig. 3. The results show that the traditional 8 μg/mL concentration experiment had little influence on cell survival. Contrarily, the 24-h cell survival rate decreased to 40% when the concentration of polybrene increased to 16 μg/mL. This suggests that the tolerance of RSCs for polybrene is reduced when the concentration of polybrene is greater than 8 μg/mL.

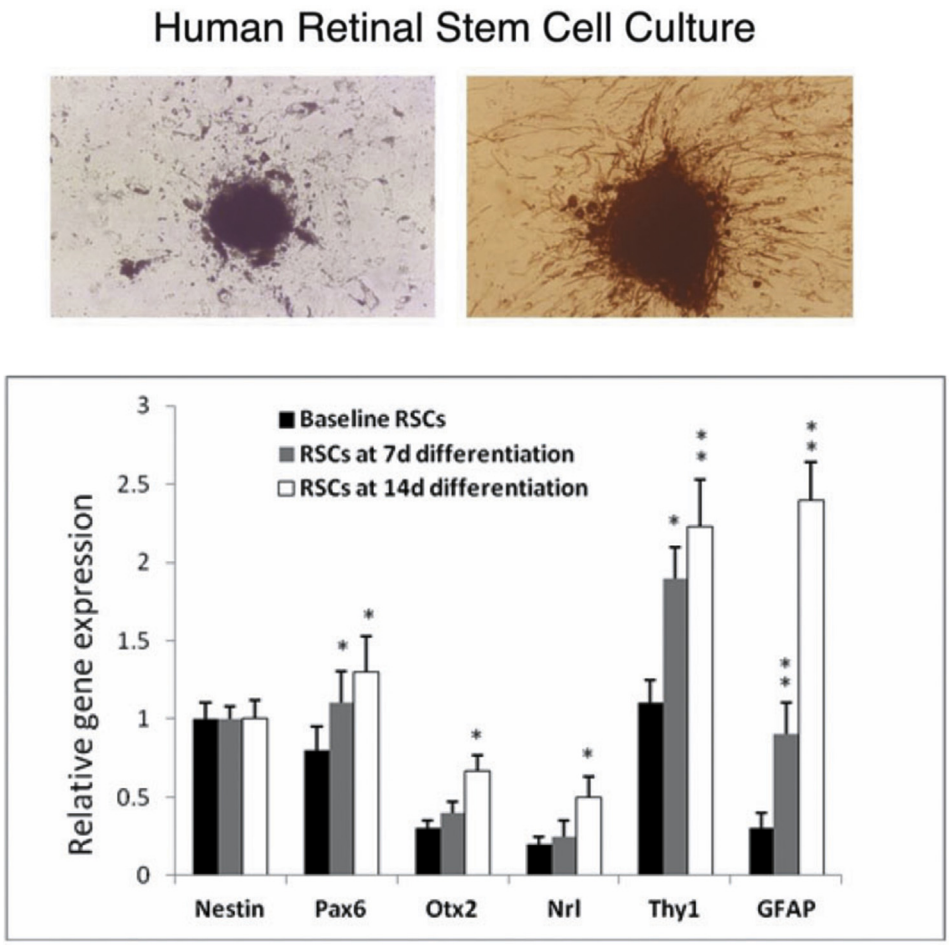

Fig. 1. Cultivation of retinal stem cell (pigment/non-pigment) from human eyes (Ciliary body zone). Isolation and cultivation of RSC neurosphere from adult human (37 y/o) eyes using serum-free medium (EGF & bFGF) condition. Cells within the sphere colony continue to proliferate and generate relatively large colonies of neurospheres (non-pigmented). The pigmented neurospheres could be maintained in vitro serum free-bFGF culture system for eight months. Self-renewal of human RSCs is seen. After seven days in vitro culture in the absence of EGF and in the presence of 1% FCS, the progression of the morphological appearance of retinal pigmented neurospheres (RPNS) revealed that RPNSs were budding and differentiating into dendritic-like neurons and axons. Spheroid-like bodies would generate the mature retinal neurons, and the gene expression features appeared to be consistent with the process of generating the mature retina from RSCs.

Effect of polybrene concentration on retroviral transduction under USWF exposure (fixed exposure time of 5 min).

RSCs with different concentrations of polybrene were exposed to the 20 Vp-p and 25 Vp-p USWF for 5 min with the experimental results shown in Fig. 4(A) and (B), respectively. The results show that the efficiency of MSCV infection in RSCs was enhanced with increased polybrene concentration. Compared to chambers which were not treated with USWF, the percentages of GFP positive RSCs with 0, 0.8, 2, and 4 μg/mL of polybrene and exposed to 20 Vp-p of USWF were not significantly different. Nevertheless, the percentage of GFP positive RSCs markedly increased after exposure to 20 Vp-p of USWF for 5 min in chambers with 8 μg/mL of polybrene.

In contrast, there was a significant enhancement of the efficiency of MSCV infection when RSCs with 2, 4 and 8 μg/mL polybrene were exposed to 25 Vp-p USWF for 5 min. Our results also showed that the percentage of GFP-positive cells did not increased in parallel with the increasing concentration of polybrene in 2, 4 and 8 μg/mL groups. RSCs in the 25 Vp-p USWF for 5 min seem to achieve the same transduction effect with lower polybrene concentration (Fig. 4).

3.4. The effect on gene delivery after different exposure times

We also exposed RSCs to 20 Vp-p and 25 Vp-p of USWF for various durations. When these RSCs were exposed to USWF for no more than 5 min, a positive correlation between the efficacy of MSCV transduction and exposure duration was found when the energy of USWF was either 20 Vp-p or 25 Vp-p. Nevertheless, a decrease of eGFP-expressing RSCs was found if the duration extended to more than 5 min (Fig. 5). Our results showed a significant impact on the ratio of GFP-a positive cells after exposure to 20 Vp-p and 25 Vp-p USWF for 5 min (30–40%). The efficacy of MSCV infection was larger with 25 Vp-p USWF than with 20 Vp-p USWF.

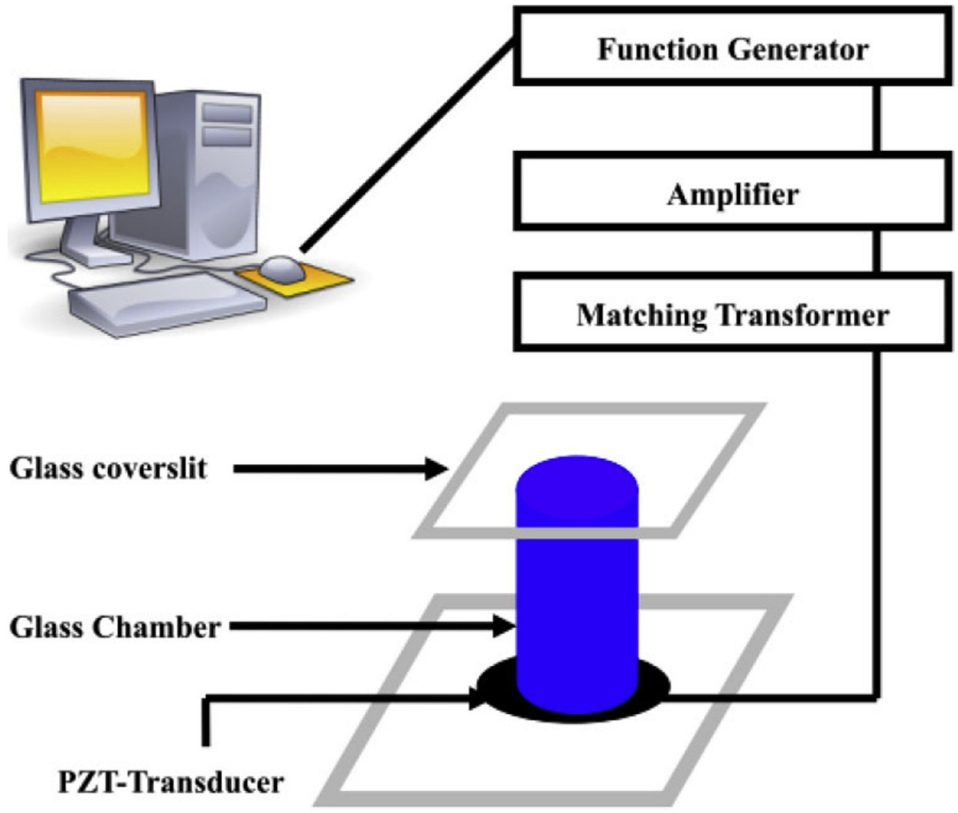

Fig. 2. Acoustic apparatus.

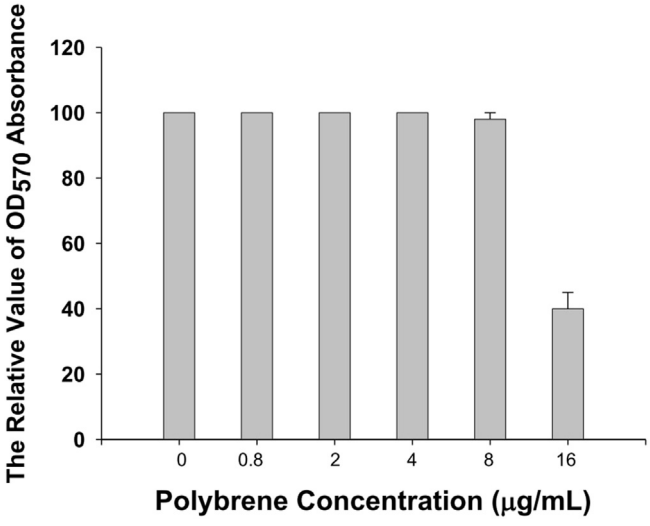

Fig. 3. RSCs in different concentrations of polybrene were tested at 24 h for survival. The cell culture medium containing different concentrations of polybrene at 24 h was measured by MTT assay are shown. The 0 μg/mL to 8 μg/mL concentration displayed little change in cell viability; at 16 μg/mL, cell viability was substantially reduced.

4. Discussion

RSCs-based therapy is a possible solution for irreversible cell loss in various retinal diseases.[9] However, some obstacles need to be overcome when applying stem cells in clinical therapy. These include insufficient amount of desired cell types, maintaining gene stability during culture, and preventing tumorigenesis.[10] Studies have shown that introducing selected genes into the target stem cells using gene therapy methods could overcome some of these problems. Moreover, due to the self-renewal feature of stem cells, the need for repeated cycles of gene therapy can be reduced. However, one of the major obstacles is the low efficiency of gene transfer to stem cells.[11]

In the past decade, several physical approaches have been exploited to improve the efficiency of retrovirus-mediated gene transfer due to low retroviral titer. The physical forces employed to increase the encounter frequency between cells and retroviruses are gravitational, convective, and magnetic. It has also been found that ultrasonic cavitation can increase extracellular molecule movement into cells, such as drugs, DNA, and virus particles.[12,13] However, the cavitation effect, in addition to increasing extracellular molecule movement into cells, may also cause cell tissue damage.[14] The main reason is the sound field of vertical vibration of the pressure caused by micro-bubbles, but the micro-bubbles also affect the sound field, causing the sound waves to repeatedly swell and shrink.[15] The center of these micro-bubbles produce high temperatures causing them to break by fission, followed by the release of a large amount of energy and causing a shockwave in the surrounding temperature. This will eventually lead to the generation of free radicals and physical injury.[16]

USWF has shown the ability to enhance the efficacy of retroviral infection. The USWF was identical in the entire system because of the dynamic equilibrium of the medium.

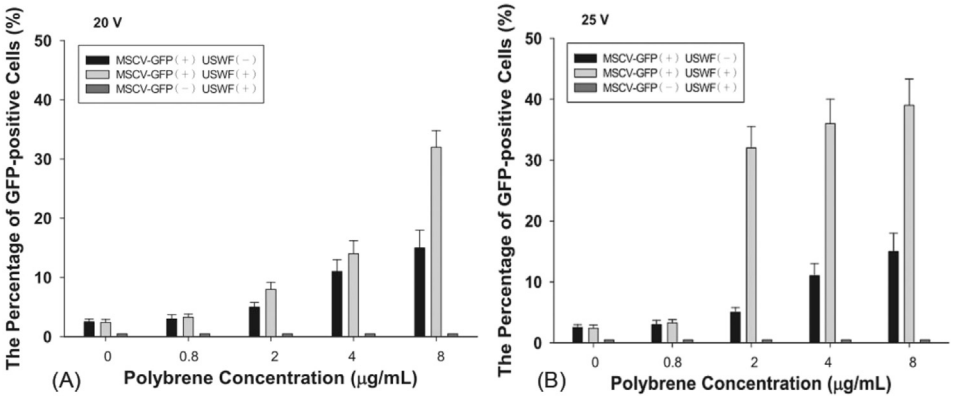

Fig. 4. (A) The effect of different polybrene concentrations with fixed exposure time of 5 min on retroviral transduction of RSCs under 20 Vp−p USWF exposure. (B) The effect of different polybrene concentrations with fixed exposure time of 5 min on retroviral transduction of RSCs under 25 Vp−p USWF exposure.

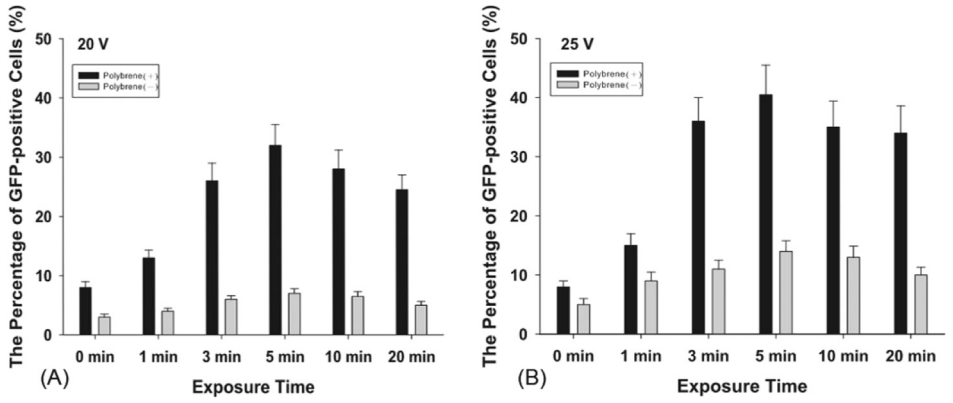

Fig. 5. The effect of different exposure times (20/25 Vp-p) on gene delivery. 20 Vp-p and 25 Vp-p drive ultrasonic standing waves were used at different times for the experimental variables, with experimental results being shown in Figure 5 (A) and (B). Both in the 20 Vp-p and 25 Vp-p USWF, the effect of MSCV transduction RSCs with increasing exposure time increased. Nevertheless, the exposure time of more than 5 min resulted in a decrease in eGFP-expressing RSCs with increasing exposure time. At the same time, we can observe the significant impact on the ratio of GFP-a positive cells for 5 min (30−40%) in 20 Vp-p and 25 Vp-p USWF. The USWF for MSCV infection of RSCs significantly enhances the appeal of the results, especially the 25 Vp-p drive.

When the ultrasound intensity exceeds a certain threshold, it will produce micro-bubbles that are generated by the change in acoustic vibration. In the USWF, there will be neither micro-bubbles nor cavitation effects in the medium. In this study, a primary radiation force generated by 1-MHz ultrasonic standing waves was harnessed to see if retroviral efficiency could be augmented. It is quite reasonable that the larger micro-particles (i.e., cells) form the bands in the USWF first and the smaller micro-particles then aggregate around the bands using the larger micro-particles as seeds. Another important advantage of USWF-mediated retroviral transfer in comparison with other physical approaches is that it is suitable for suspension cells; thus, it is easy to use in large-scale operations.

Three main parameters were examined in our study. The first was the concentration of polybrene. The positively charged polymer can help the RSCs to overcome the electrostatic repulsion once microstreaming forces have dragged retroviruses into close proximity of the preformed cell bands, thereby promoting the binding of the retrovirus to the surface of the target cells. It also offers shields and plays an important role in retroviral transduction of USWF. Our results showed that the efficiency of MSCV infection improved with increasing of concentration of polybrene. However, the cells would exhibit cytotoxic properties if the amount of polybrene was too high.

The second parameter was the energy of the ultrasound. Our study found that the survival rate of RSCs remained unchanged after the exposure to either 20 Vp-p or 25 Vp-p of USWF for 5 min. In contrast, the survival rate decreased after exposure to 30 Vp-p USWF. A cell band formed in the field when the RSCs exposed to 30 Vp-p of USWF. We believed that the vibration was more intense in RSCs of cell bands and decreased the short- and long-term survival of these RSCs. In the presence of 2 μg/mL polybrene, fluorescence intensity of eGFP detected in RSCs under 25 Vp−p and 20 Vp−p USWF exposure was roughly six-fold and 1.2-fold, respectively, higher than those without USWF. It seems that lower polybrene concentration could achieve the same effect as traditional virus transfection when RSCs were exposed to 25 Vp−p USWF. These results suggest that USWF exposure could improve the efficacy of MSCV transfecting RSCs.

Finally, the exposure time of USWF was demonstrated to play an important role. We exposed RSCs to 20 Vp-p and 25 Vp-p of USWF for various durations. When these RSCs were exposed to USWF for no more than 5 min, a positive correlation between the efficacy of MSCV transduction and exposure duration was found regardless if the energy of USWF was 20 Vp-p or 25 Vp-p. Nevertheless, a decrease of eGFP-expressing RSCs was found if the duration extended to more than 5 min (Fig. 5). Out results showed a significant impact on the ratio of GFP-a positive cells after exposure to 20 Vp-p and 25 Vp-p USWF for 5 min (30−40%). The efficacy of MSCV infection was enhanced more strongly with 25 Vp-p USWF than with 20 Vp-p USWF. Longer exposure periods could provide more binding opportunities between cells and retroviruses. However, the effect is limited. We found that 5 min was the optimal time under 1 MHz and 25 Vp−p of output energy with 106 cells/ml. We found that the proportion of eGFP-expressing RSCs would decreased when we extended the duration of exposure to more than 5 min. This conflicted with the principle that the longer the USWF exposure time, the greater the total encounters between cells and retroviruses should be.

Target site specificity and transfection efficiency are two main problems to overcome in current gene-based therapies and ultrasound has recently been applied in gene and drug delivery because it can elevate both transfer efficiency and organ or tissue specificity.[17] Though ultrasound-targeted microbubbles have gained increasing attention as a carrier in these attempts, the negative effects on nearby tissue is still unknown. The main applications of microbubble-assisted gene or drug therapy are focused on cardiovascular and central nervous systems because of their special anatomical structures.[18] However, microbubbles in ocular treatments generally require prompt parameter adjustments to achieve adequate drug release time and dose without causing damage to nearby ocular tissue.

In this study, we applied an USWF system with MSCV in RSCs to avoid the above disadvantages. Our results demonstrated that acoustic wave fields established by standing waves could enhance gene transduction efficiency of MSCV to human RSCs. Optimal operating parameters, including ultrasound exposure time, polybrene concentration, and acoustic intensity, were investigated to gain the highest efficiency of retroviral transduction. Results in this study showed retroviral gene transfer was improved by nearly 10% without apparent cell death. In addition, the apoptotic activity of retinal stem cells under the exposure of acoustic waves was explored to guarantee the sustainability of RSCs for therapeutic use.

Acknowledgments

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