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Original Article

Vascular gene transfer and drug delivery *in vitro* using low-frequency ultrasound and microbubbles

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Aim: To determine the effects of ultrasound exposure in combination with a microbubble contrast agent (SonoVue) on the cellular uptake and delivery of drugs/genes into human umbilical vein endothelial cells (HUVECs) as well as their biological effects on migration.

Methods: HUVECs in suspension were exposed to pulsed ultrasound with a 10% duty cycle in combination with various concentrations of a microbubble contrast agent (SonoVue) using a digital sonifier at a frequency of 20 kHz and an intensity of 3.77 W/cm^2 on the surface of a horn tip. Cell culture inserts were used to determine the cell migration ability.

Results: Exposure to pulsed ultrasound resulted in enhanced green fluorescent protein (EGFP) gene transfection efficiencies ranging from 0.2% to 2%. The transfection efficiency of HUVECs was approximately 3-fold higher in the presence of SonoVue than in its absence at the effective exposure time of 6 s. For drug delivery to HUVECs using ultrasound, the delivery efficiencies of a low-molecular-weight model drug (TO-PRO®-1, M_W 645.38) were significantly higher when compared to drug delivery without ultrasound, with a maximum efficiency of approximately 34%. However, the delivery efficiencies of a high-molecular-weight model drug (Dextran-Rhodamine B, M_W 70 000) were low, with a maximum delivery efficiency of nearly 0.5%, and gene transfection results were similarly poor. The migration ability of HUVECs exposed to ultrasound was also lower than that of the control (no exposure).

Conclusion: The use of low-frequency and low-energy ultrasound in combination with microbubbles could be a potent physical method of increasing drug/gene delivery efficiency. This technique is a promising nonviral approach that can be used in cardiovascular disease therapy.

Keywords: ultrasound exposure; human umbilical vein endothelial cells; microbubble; gene transfection; drug delivery; cell migration

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Introduction

Gene therapy and drug delivery are promising tools for the treatment of several conditions, such as cancer and cardiovascular diseases[1, 2]. However, current clinical applications are hampered by the absence of safe and efficient systems for local gene/drug delivery to specific tissues or organs. For gene therapy, nonviral gene delivery can be performed by the direct injection of DNA, but such approaches are generally associated with low transfection efficiencies and only transient expression of the gene product. Viral vectors significantly increase transfection efficiency because of the specific viral machinery that has evolved to introduce foreign DNA into mammalian cells. However, viral proteins elicit an unwanted immune response within the targeted hosts or tissues[3].

For many decades, ultrasound technology has been widely used for diagnostic imaging in clinical fields without any significant adverse side effects. In recent years, there has been intense interest in developing an efficient, simple way to deliver DNA or drugs into cells[4]. It has been shown that sonication (ultrasound) can alter the transient permeability of plasma membranes to facilitate uptake[5]. Compared with other direct DNA delivery methods, such as particle gun bombardment, electroporation, and microinjection, sonication may be simpler to carry out. Sonication, however, can cause cell damage or even rupture. If sonication is used to facilitate uptake, it is important to optimize the conditions or parameters without causing cell damage. Mild ultrasound irradiation has proved to be an efficient method of transfecting genes into animal cells and tissues *in vitro* and *in vivo*[6, 7].

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Encapsulated gas microbubbles (usually 2–8 μm in diameter) have been used as ultrasound contrast agents in medical ultrasound imaging for several decades. Recently, ultrasound-induced microbubble destruction has been proposed as a new technique for the local delivery of genes and drugs to specific target tissues, including the heart[8–10]. Ultrasound can cause a transient, nonlethal perforation of the capillary and cell membranes because of cavitation effects and thereby improve transfection and drug delivery efficiencies. In many studies, ultrasound-enhanced gene/drug delivery techniques combined with microbubbles encasing an expression vector have been shown to enhance gene transfection and drug delivery[10, 11]. These studies focused mainly on cancer and the vascular system. The cavitation of microbubbles under an ultrasound field will lead to an increase in microvascular permeability and induce small gaps between capillary endothelial cells due to the cavitation energy, which will enable drugs to leak from the vessel and reach the target location[9, 11, 12]. Recently, Ohta *et al* used microbubble-enhanced sonoporation to achieve ectopic and transient gene expression in several embryonic organs, including embryonic chick limb bud mesenchymes[13]. Recently, ultrasound has also been used as an external trigger for the controlled release of drugs[14, 15]. These studies showed that ultrasound is one of the most promising external triggers for pulsatile delivery, and the release rate of the incorporated drug can be altered by external application of ultrasound radiation[15]. Although ultrasound is widely applied in gene transfection and drug delivery, different ultrasound exposure modalities or setups will result in different delivery efficiencies of DNA or drugs[3, 16, 17].

In this research, gene transfection and drug delivery by sonoporation were examined using cultured vascular endothelial cells (HUVECs) with a microbubble contrast agent (SonoVue). Various parameters and experimental conditions for sonoporation to introduce exogenous molecules (DNA and drugs) into HUVECs were used and discussed. The feasibility and mechanisms of gene and drug delivery to HUVECs were tested in our exposure setup. The cytoskeleton arrangement and migration ability of HUVECs after ultrasound exposure were also investigated.

Materials and methods

Reagents

Cell culture medium RPMI 1640, HAT (hypoxanthine, aminopterin, and thymidine) supplement, and newborn calf serum (NCS) were purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium (MTT), and 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (HEPES) were provided by Amresco (Cleveland, OH, USA). Trypsin, penicillin and streptomycin were obtained from Sigma-Aldrich (St Louis, MO, USA). SonoVue microbubbles were purchased from BRACCO (Milan, Italy). TO-PRO®-1 and Dextran-Rhodamine B were obtained from Molecular Probes (Invitrogen). All other chemicals were of analytical grade and used without further purification.

Cell culture

The HUVEC line EA Hy926 (passages 5-8) was obtained from the Institute of Biomedical Engineering of the Western China Medical College (Sichuan, China). This cell line, a hybridoma of HUVECs and the human epithelial cell line A549, has been shown to retain a number of properties of endothelial cells, including the production of human factor VIII-related antigen. The cells were cultured in RPMI 1640 containing 20 mmol/L HEPES, 10% NCS, 2% $NaHCO_3$, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2% HAT supplement at a temperature of 37 °C in a humidified 5% CO_2 atmosphere. Confluent cells were trypsinized and seeded at a density of 1×10^5 cells/well (1 mL) in 12-well plates (Corning, NY, USA). The culture medium was removed when the cells reached 70-80% confluence, and 2 mL of new culture medium containing enhanced green fluorescent protein plasmid (pEGFP-N1, containing an EGFP reporter gene) or model drugs (TO-PRO®-1 or Dextran-Rhodamine B) were added to the wells. The samples were then used for ultrasound exposure experiments.

Ultrasound exposure

Ultrasound was generated using a 20-kHz digital sonifier S-250D (Branson Ultrasonics, Danbury, CT, USA) at a power of 3 W with an intensity of 3.77 W/cm^2 on the surface of a tapered microtip with a diameter of 12.64 mm (Figure 1). The vibration amplitude was 9%, and the effective exposure time ranged from 0 to 15 s (total exposure time 0–150 s) with a 10% duty cycle (ultrasound exposure: on for 0.1 s and off for 0.9 s). The pEGFP-N1 plasmid (or model drug), with or without SonoVue, was added to the cells that were exposed to ultrasound. The control samples received either pEGFP-N1 plasmid (or model drug) only or pEGFP-N1 plasmid plus SonoVue without ultrasound exposure. The exposure experiments were carried out on HUVEC monolayer cultures in 12-well plates.

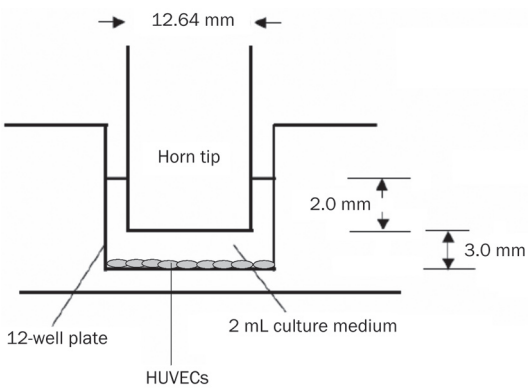


Figure 1. Schematic ultrasound exposure system with digital sonifier at 20 kHz for HUVECs in suspension.

Cell viability assay

Cell viability was determined by the MTT colorimetric assay. This assay depends on the ability of cells to metabolize a

yellow tetrazolium salt, MTT bromide dye, to a dark-blue formazan product. Cells (1×10^4) were suspended in 200 μL of culture medium and seeded into 96-well plates and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Then 20 μL of MTT (0.5 mg/mL) was added into the wells, followed by further incubation for 4 h at 37 °C. After the supernatants were removed, 150 μL of DMSO was added to dissolve the formazan crystals formed. Absorbance was measured colorimetrically at 570 nm. Cell viability was calculated as the percentage of the absorbance of treatment groups relative to that of the control.

Gene/drug delivery assay *in vitro*

Cells cultured 24 h after exposure were washed twice in phosphate-buffered saline (PBS), after which the qualitative analysis of EGFP gene expression or model drug delivery into the cells was observed under an inverted fluorescence microscope (TE-2000U, Nikon, Japan). Flow cytometry analysis was used to quantify the efficiencies of gene transfection and drug delivery into the HUVECs. Samples were analyzed on a FACSCalibur machine (Becton Dickinson, Franklin Lakes, NJ, USA) for fluorescence, and 30 000 events were captured for each sample.

Cell migration assay

The HUVEC migration experimental system utilized Millicell® cell culture inserts (Millipore, Billerica, MA, USA) containing PET membranes with a pore size of 8 μm. Twelve-well plates were used for the HUVEC migration after ultrasound exposure[18]. Each well contained 1.5 mL of RPMI 1640 culture medium, and 500 μL of the exposed cell suspension was added to each insert. The cells were cultured for 2 to 12 h in a 5% CO_2 incubator at 37 °C. The culture medium was removed, and cells on the PET membranes that had not migrated were scraped off using a cotton stick. The cells that migrated through the pores (determined by adhesion to the outer side of the PET membrane) were fixed for 30 min using a mixed solution (5 mL) of acetic acid and methyl alcohol (1:3, *v/v*) after the inserts were washed twice with PBS. The PET membranes were taken out using a scalpel and dyed for 10-15 min with Giemsa solution (0.9%) on a glass slice. The excess Giemsa solution was washed with Milli-Q water three times. The numbers of dark blue migrated cells were quantified by cell counting under an inverted microscope from more than ten random microscopic fields (magnification 60×) for each sample.

Presentation of data and statistical analysis

All data are presented as mean±standard deviation (SD). The significance of any differences between experimental groups was assessed using two-tailed Student's *t*-test. Differences between groups were considered significant at $P<0.05$ or $P<0.01$.

Results and discussion

Effects of ultrasound and microbubbles on HUVEC viability

The numbers of viable HUVECs decreased linearly with ultrasound exposure time, as shown in Figure 2A, but the viability was still more than 75% even at the effective exposure time of 12 s. The mechanical and physical forces of the ultrasound exposure will destroy the cell if the intensity and exposure time are beyond the tolerance limit of the culture and can even induce cell lysis leading to death[19, 20]. Our data also indicate that the SonoVue microbubbles had a minimal effect on the viability of HUVECs (Figure 2B) as it was measured at more than 90% even at high concentrations (40%, *v/v*). For a SonoVue concentration of 10% (*v/v*), the cell viability observed was very similar to that of the control (0%, *v/v*), indicating that 10% SonoVue microbubble treatment has almost no effect on the viability of HUVECs. Thus, this concentration was used in the succeeding experiments. In previous studies, microbubbles exhibited side effects when injected into patients[21–23]. Our experiments demonstrated that the side effects of SonoVue microbubbles were not as evident as those of other reported ultrasound contrast media. It was also demonstrated that SonoVue is an ideal microbubble for gene/drug delivery.

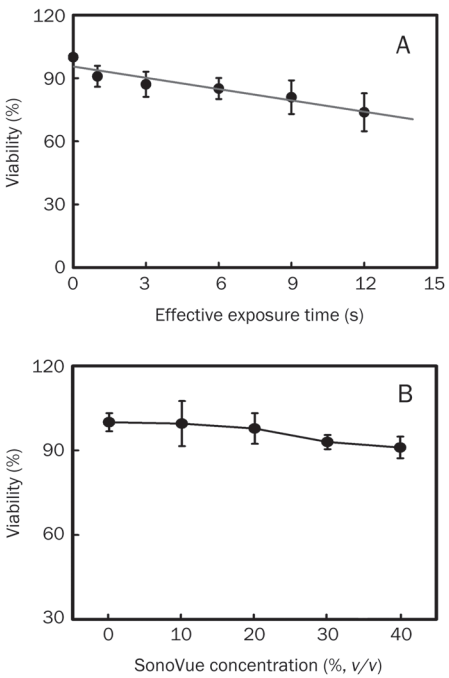
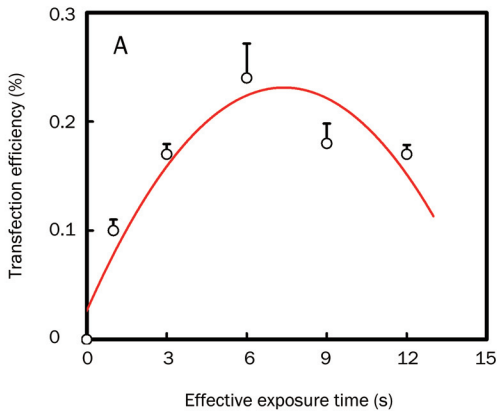


Figure 2. (A) Cell viability *versus* effective ultrasound exposure time with 10% duty cycle in the presence of 10% (*v/v*) SonoVue microbubbles. (B) Cell viability of HUVECs *versus* various SonoVue concentrations without ultrasound exposure. Each point is the mean of three independent experimental data.

Gene transfer into HUVECs using ultrasound and microbubbles

After ultrasound exposure, the cells that expressed EGFP were observed under a fluorescence microscope. The EGFP-N1 plasmid DNA was successfully transferred into HUVECs, as shown in Figure 3C. The transfection efficiencies, defined as the ratio of the transfected cells to the total cell number, first



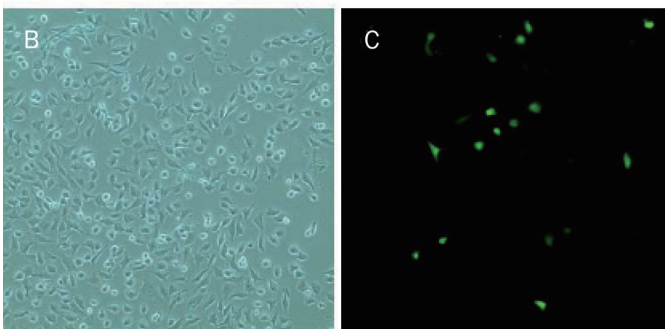


Figure 3. (A) Transfection efficiencies of HUVECs versus various effective ultrasound exposure times in the presence of 2 μg/mL EGFP-N1 plasmid. Each point is the mean of five independent experimental data; (B) Bright-field micrograph of cultured HUVECs; and (C) Fluorescence micrograph of cells showing EGFP expression following ultrasound exposure of 6 s (magnification×15).

increased with the effective exposure time, and then decreased when the exposure time was beyond 6 s. In other words, there was a transfection efficiency peak at 6 s of exposure (Figure 3A). The increase in transfection efficiencies at the first stage was due to the fact that more plasmid DNA could enter the HUVECs with increased exposure time. However, the number of dead cells was higher than that of transfected cells when the effective exposure time was longer than 6 s. As a result, the transfection efficiencies decreased in the second stage (>6 s). This phenomenon was also observed in our previous studies on HeLa-S3 cells[24]. The number of dead cells and transfection efficiency increased with exposure time. However, cell death or lysis predominated, leading to a decrease in transfection efficiency. Some cells may have successfully incorporated the plasmid following ultrasound sonoporation, but they could not express the transferred gene because they died quickly.

Figure 4 shows the effects of increasing SonoVue microbubble concentrations on the transfection efficiencies of HUVECs. After 6 s of ultrasound exposure, the transfection efficiencies were quantified by flow cytometry. The transfection efficiencies were relatively high in the presence of 10% and 20% (*v/v*) SonoVue microbubbles, but there was no significant difference observed between the two concentrations. Interestingly, a

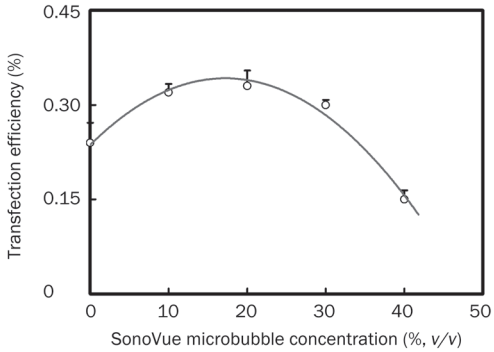


Figure 4. Effect of SonoVue microbubble concentrations on transfection efficiencies of HUVECs following 6 s of ultrasound exposure with 10% duty cycle in the presence of 1 μg/mL EGFP-N1 plasmid. The 0% Sonovue indicates the control of "no SonoVue". The data were obtained from three independent experiments.

high concentration of SonoVue (40%, *v/v*) had no synergistic effect on gene transfection enhancement with ultrasound but caused a further decrease in gene transfection efficiency to levels even lower than those of the control sample (0%, *v/v*). One explanation for this phenomenon may be that the high microbubble concentration caused more cell death when combined with the ultrasound field. Thus, we compared the transfection efficiencies of HUVECs in the presence or absence of 10% (*v/v*) SonoVue microbubbles. It was found that microbubbles at an appropriate concentration (10%, *v/v*) could significantly enhance cell transfection efficiency (Figure 5). The transfection efficiency of HUVECs in the presence of microbubbles with 6 s of ultrasound exposure (the peak) was approximately 3-fold higher than in the absence of microbubbles, which also demonstrated that the ultrasound microbubbles had a synergistic effect on gene transfection. However, SonoVue microbubbles are destroyed by ultrasound. This destruction generated high-energy microstreams or microjets that produced shear stress surrounding the cell membranes and increased permeability[11, 25]. The increase in permeability was probably due to transient "pores" in the plasma mem-

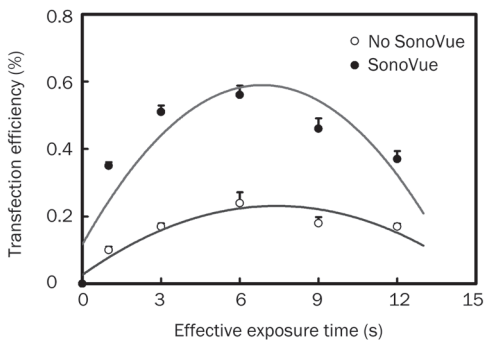


Figure 5. Transfection efficiencies of HUVECs following ultrasound exposure with 10% duty cycle with or without 10% (*v/v*) SonoVue microbubbles in the presence of 1 μg/mL EGFP-N1 plasmid. The data were obtained from three independent experiments.

brane[26]. Ultrasound with microbubbles could potentiate gene delivery by creating transient nonlethal perforations in cell membranes to aid ingress of molecules (DNA or other drugs) into the cells ("sonoporation"). The rapid vibration of microbubbles on the surfaces of the cells, or microstreaming, likely allows for the enhanced delivery of DNA across the cell membrane[11, 12, 27].

For gene transfections using ultrasound, another important factor affecting efficiency is plasmid concentration. Therefore, the transfection of HUVECs using various concentrations of EGFP-N1 plasmid was also studied. The transfection efficiencies increased with the plasmid DNA concentration, as shown in Figure 6A. This tendency could also be observed in the fluorescent images (Figure 6B). The number of cells that expressed EGFP in the presence of 8 μg/mL of plasmids is significantly high. It is reasonable to assume that there are more chances for plasmid DNA entry into cells if the plasmid concentration is high when the cells are exposed to ultrasound.

The gene transfection data shown in Figures 3–6 reflect transfection efficiencies that are not at the desired levels. In 2008, Wang *et al*[28] reported that the transient transfection rate of HUVECs was approximately 20.3% using a recombinant plasmid (pEGFP-KDR-TK) under 1.9 MHz continuous ultrasound with 80 mW/cm^2 output intensity. In our opinion, it is reasonable that different ultrasound exposure setup and exposure modalities can greatly affect gene transfection efficiency. Other parameters, such as exposure intensity, frequency, type of cell culture plate, cell type, and plasmid type and size, will also remarkably affect gene transfection efficiency. Our data showed that the technique of immersing the microtip directly into the cell culture medium was not preferable to the reported methods of Wang *et al*[28]. Further investigation is still needed to reach acceptable levels of gene transfection in HUVEC cultures.

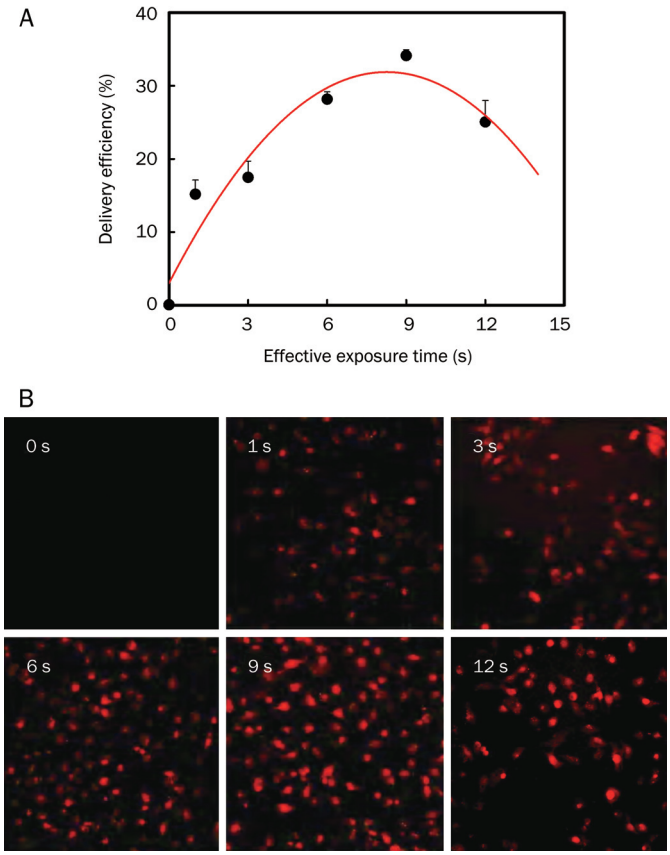


Figure 7. (A) Cellular uptake (delivery efficiency) of fluorescent molecules of TO-PRO®-1 (as the low molecular weight model drug) into HUVECs following ultrasound exposure in the presence of 10 μg/mL TO-PRO®-1. Each point is the mean of three independent experimental data. (B) Fluorescence micrographs of the model drug delivery into HUVECs (magnification ×20).

Delivery of model drugs into HUVECs using ultrasound

Ultrasound can also be used in drug delivery, particularly for cardiovascular disease therapy. Figure 7 shows that TO-PRO®-1 molecules can be delivered into HUVECs with high efficiency. TO-PRO®-1 is a nucleic fluorescent dye with a low molecular weight of 645.38 daltons and has the unique property of being unable to permeate intact cell membranes. In this study it was used as a model drug to quantify intracellular uptake into viable cells. The drug delivery efficiency was very similar to the observed efficiency of gene transfer to HUVECs.

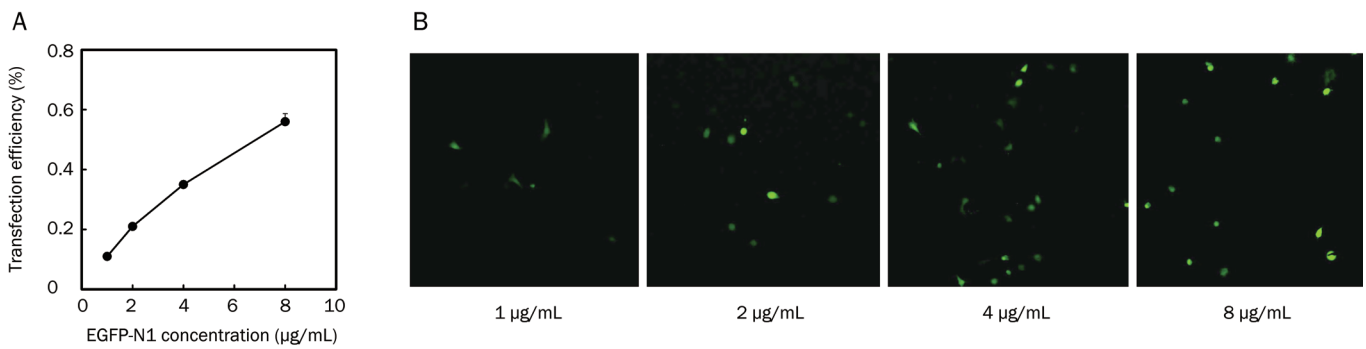


Figure 6. (A) Transfection efficiencies *versus* plasmid concentrations of EGFP-N1 under an effective exposure time of 6 s. Each point is the mean of three independent experimental data. (B) Fluorescence micrographs of EGFP expression in the presence of plasmid at various concentrations (magnification ×15).

However, the peak of delivery was found at an effective exposure time of 9 s, whereas for gene transfer the peak was found at an effective exposure time of 6 s. Notably, the delivery efficiencies of this model drug with a low molecular weight were significantly higher than that of plasmid DNA. The maximum efficiency was approximately 34%—more than 150-fold that of plasmid DNA transfection. To determine whether this phenomenon was also observed in the case of other drugs, a high-molecular-weight model drug, Dextran Rhodamine B (70 000 Dalton), was used in our experiments (Figure 8). Interestingly, the delivery efficiencies were very low, and the maximum transfection efficiency was 0.5% at an effective exposure time of 15 s, which was very similar to the delivery efficiency of EGFP-N1 plasmid DNA. One possible reason for this result is that small molecules can easily enter the cells following ultrasound exposure, which opens some transient "pores" on the cell membrane, whereas the plasmid DNA and Dextran Rhodamine B are macromolecules that diffuse poorly into the cells[29, 30].

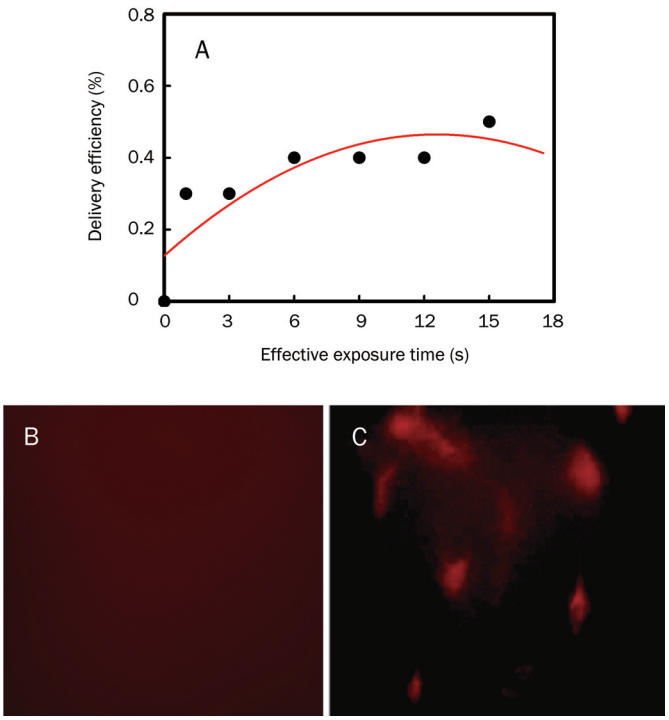


Figure 8. (A) Cellular uptake (delivery efficiency) of fluorescent molecules of Dextran-Rhodamine B (as the high-molecular-weight model drug) into HUVECs following ultrasound exposure in the presence of 40 μg/mL Dextran-Rhodamine B. Each point is the mean of three independent experimental data. Representative fluorescence micrographs showing model drug (Dextran-Rhodamine B) delivery into HUVECs exposed to ultrasound for 12 s (C) and into control cells (no ultrasound exposure) (B) (magnification ×60).

Effects of ultrasound on migration of HUVECs

To further investigate the biological effects of ultrasound exposure on HUVECs, cell migration ability was assayed *in vitro* using Millicell® cell culture inserts. Figures 9A and 9B show the transmembrane migration of the control (no exposure) and exposed cells, respectively. It was found that no cell migration occurred during the first 2.5 h, and a few migrated cells were

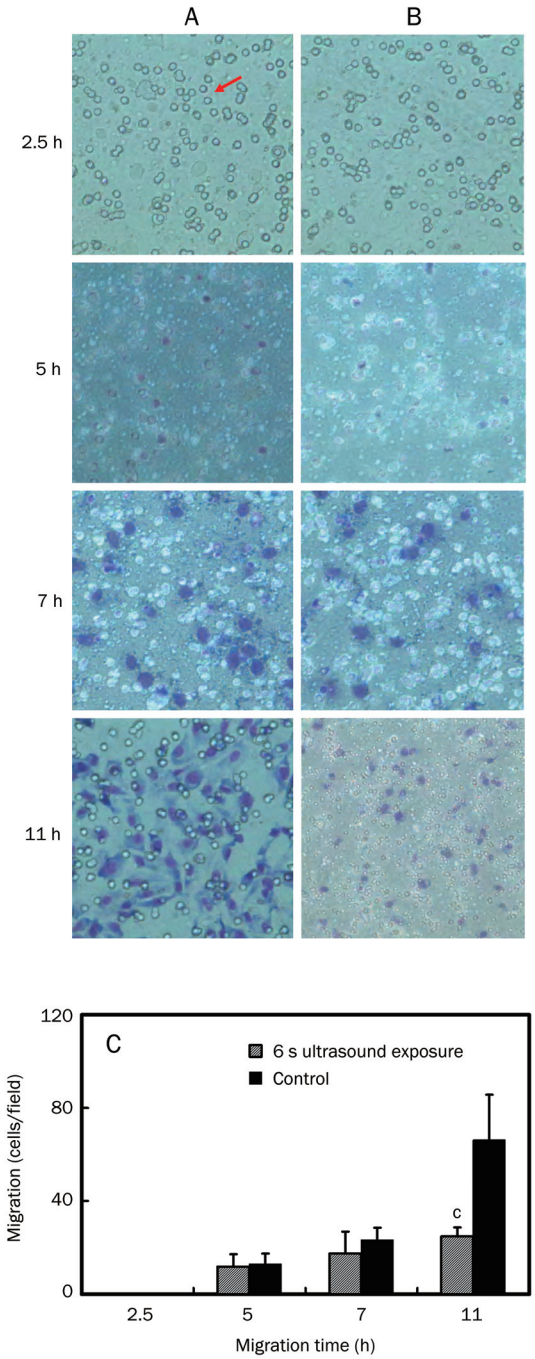


Figure 9. Migration comparison of HUVECs after ultrasound exposure (6 s) (B) and the control (no ultrasound exposure) (A). The cells stained dark-blue are the transmembrane-migrated HUVECs. The small open circles (marked by an arrow) are the pores of the PET membrane of Millicell® Cell Culture Inserts (magnification 60×). (C) The numbers of migrated cells were quantified by cell counting from 10 microscopic fields for each culture time point. The data were obtained from three independent experiments. $^{c}P<0.01$ *vs* control at 11 h.

observed after incubation for 5 h. This lack of migration is due to the time that cells need to adhere to the PET membranes before migration could occur. Many migrated cells (stained dark blue) were observed, particularly after 11 h of incubation, but the number was still lower than that of the control. It could be concluded that ultrasound exposure decreased HUVEC mobility and migration. Given the importance of the cytoskeleton to cell adhesion and migration, there is a possibility that the cytoskeleton of the cells exposed to ultrasound would be disordered. Aside from the cytoskeleton, it is also possible that the activities of some proteins or enzymes in the cells were also affected by ultrasound. In our previous study, ultrasound exposure was shown to affect some protein activity, for instance, that of Ca^{2+}-ATPase[31]. To fully understand the molecular mechanisms and the biological effects of ultrasound, further investigations are necessary.

Summary

Although the primary effect of ultrasound exposure *in vitro* is cell lysis induced by ultrasound cavitation, sublethal damage may also occur with the passage of some molecules (*eg*, DNA and drugs). The transient permeability of cell membranes will lead to the uptake of extracellular molecules into the cells. Our study showed that sonoporation, in the presence of microbubbles, was a promising technique that permits the transfer of drugs and genes into cells. Additionally, low-frequency and low-energy ultrasound-induced destruction of microbubbles is a feasible and efficient method of vascular gene transfection and drug delivery. Although the exact mechanisms underlying efficient drug uptake and gene transfection remain incompletely understood, the rapid collapse of microbubbles during sonoporation has been considered to play a major role in drug/gene delivery into cells. It has also been speculated that the presence of microbubbles can significantly reduce the threshold of acoustic cavitation upon ultrasound exposure in a target site. At this time, the study of the application of microbubbles with ultrasound for gene/drug delivery is still currently in its infancy, particularly *in vivo*.

Acknowledgements

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Author contribution

Yi-yao LIU designed the research; Hong YANG, Zhong-hua LIU, and Chang-chun LOU performed research; Zheng-long REN and Hirokazu MIYOSHI contributed new analytical tools and reagents; Hong YANG and Yi-yao LIU wrote the paper.

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