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A Novel Approach of Low-frequency Ultrasonic Naked Plasmid Gene Delivery and Its Assessment[1]

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Objective To deliver the naked genes into cells through the bioeffects of cell membrane porous produced by low-frequency ultrasound (US) and to investigate the safety by determining the threshold of cell damage and membrane permeability. **Methods** The suspension of red cells from chickens, rabbits, rats, and S180 cells was exposed to calibrated US field with different parameters in still and flowing state. Laser scanning confocal microscopy, fluorescent microscopy, scanning electron microscopy, flow cytometry and spectrophotometry were used to examine cell morphology, membrane permeability, enzymes, free radicals, naked gene expression efficiency, threshold of cell damage and cell viability. **Results** The plasmid of green fluorescent protein (GFP) as a reporter gene was delivered into S180 cells under optimal conditions without cell damage and cytotoxicity. The transfection rate was (35.83±2.53)% (n=6) in viable cells, and the cell viability was (90.17±1.47)% (n=6). Also, malondialdehyde, hydroxyl free radical, alkaline phosphatase, and acid phosphatase showed a S-shaped growth model (r=0.98±0.01) in response to the permeability change and alteration of cell morphology. The constant E of energy accumulation in US delivery at 90% cell viability was an optimal control factor, and at 80% cell viability was the damage threshold. **Conclusion** US under optimal conditions is a versatile gene therapy tool. The intensity of GFP expression in US group has a higher fluorescent peak than that in AVV-GFP group and control group (P<0.001). The optimal gene uptakes, expression of gene and safety depend on E, which can be applied to control gene delivery efficiency in combination with other parameters. The results are helpful for development of a novel clinical naked gene therapeutic system and non-hyperthermia cancer therapeutic system.

Key words: Ultrasound gene delivery; Drug delivery; Ultrasonic bioeffects

INTRODUCTION

Ultrasound (US) has become an important and significant medical tool. It is indispensable in diagnostic medicine and has established applications in therapy. The mechanisms for US bioeffects can be broadly characterized as resulting from either thermal or nonthermal effects. The nonthermal mechanisms include radiation force effects, bulk streaming of liquids, and cavitation. It is generally considered that heat and cavitation are the two mechanisms of damage from US which may be most important[1]. US can further enhance porous effect on cell membranes and incorporate large external molecules into the cell and heal the membranes[2-4]. This process can transfer even large molecules such as DNA into living cells. Using the bioeffects of US, we can deliver drugs and genes into cells and improve the uptake of cells, which can be applied to gene therapy[5-9]. US can help to deliver DNA to specific areas in the body and to track its progression using contrast agents injected with the microbubbles of genetic material[10-12]. Researchers are also applying US to targeted drug delivery through a process called sonophoresis[13-14]. This technique uses US instead of needles to inject drugs such as insulin and interferon directly through the skin[15]. Some scientists are trying to use specially created DNA codes to treat cancerous tumors through gene therapy. While progress has been slow because of the lack of an ideal method to deliver therapeutic genes into tumor cells[16-17]. US gene delivery could provide a better solution.

The aim of this study was by using the bioeffects of cell membrane porous produced by low-frequency US to deliver naked genes into cells and to investigate the safety of US gene delivery, to determine the

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threshold of cell damage and controlling factors of cell membrane permeability. Furthermore, the safety of the technique was evaluated by changes of enzymology, morphology and other relevant factors.

MATERIALS AND METHODS

Preparation of Cell Sample

Freshly drawn blood from rabbits, rats and chickens with anticoagulants was stored at 4℃ before use and red blood cells were collected by centrifugation (Boifuge 15R, HEERAEUS Germany; 400 g, 8 minutes, 4℃), washed 3 times with phosphate-buffered saline (PBS, pH 7.4, Sigma), finally suspended in PBS at a concentration of 10% by volume. The cell suspension was stored at 4℃ and then gently diluted to 2×10^6 cells/mL before experiment. In the still experiments, 4 mL of the red blood cell suspension was added to a 10 mL airtight polypropylene centrifuge tube to have an ultrasound exposure (UE). After UE, 5% (v/v) heat inactivated fetal bovine serum (HIFS) was added to the sample and stored at 4℃ for later observation. For the experiment of flowing state, 20 mL of red blood cell suspension of rats was added to a 25 mL airtight polypropylene centrifuge tube to prepare an UE in flowing state which was produced by a small extracorporeal circulation pump of small animals (WSQ-A, Wheel Infusion Pump, Jiangsu, China).

S180 mouse tumour cells were inoculated into the abdomen of small white mice. After four days, ascites was collected by abdominocentesis and cells were harvested by centrifugation (Boifuge 15R, HEERAEUS Germany; 400 g, 6 minutes, 4℃), washed 4 times with PBS. The suspension of S180 cells was cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37℃ in RPMI-1640 media, supplemented with 10% (v/v) heat inactivated fetal bovine serum (HIFS) and 100 μg/mL penicillin-streptomycin (Sigma). One day later, cells were collected by centrifugation (200 g, 5 min, 4℃) and then suspended in RPMI-1640 media at the concentration of 10^6 cells/mL before UE. In the experiments, 4 mL of the cell suspension was put into a 5 mL polypropylene centrifuge tube and sealed to prepare for UE as aforementioned. After UE, the sample was added into 5% (v/v) HIFS and cultured for about 48 hours and examined.

Ultrasonic Exposure and Calibration

An ultrasonic naked gene delivery (UNGD) system was set up, in which an accurate multi-function power amplifier (0-100 W, adjusting the input voltage) with a signal generator (20-100 kHz, continuous-wave) worked in a continuous-wave (CW) manner to drive an unfocused, circular piezoelectric crystal US transducer (PZT, the sound source, 35.1 kHz) with an active element of 2.5 cm in radius. The electric power with a manostat was applied to the system. The frequency of signals was matched with the resonant frequency of transducer. An inductor was connected in parallel with the transducer to optimize its performance. A frequency meter (SD4040 Tachometer) was used to calibrate the frequency.

The experiment condition should be specified with adequate accuracy to produce valid results, thus the calibration of acoustic power was important. The ultrasonic wave generated at low frequency (35.1 kHz) was not a good plane wave, the intensity at point of cell exposure was not equal to that on the surface of the source. Thus, US exposed to cells employed a calibrated hydrophone (Reson, Goleta, CA) to measure the acoustic pressure directly to which the cells were exposed at room temperature (24 ± 1)℃ and to calculate the intensity with a standard method[15]. Furthermore, a portable advanced US power meter (UPM-DT-100, OHMIC) was calibrated for low frequency using the hydrophone to evaluate power of the transducer. In the practice of gene therapy, it was impossible to measure acoustic pressure with a hydrophone in human body. Using a calibrated portable advanced US power meter to evaluate the intensity was a very useful method in reality. The US intensity delivered was controlled by adjusting the input voltage. The system could control the frequency, intensity and total exposure time.

The UE apparatus consisted of a transducer was fixed in a double layer acrylic tank (Inner size 20 cm×15 cm×15 cm). The outer layer is a cooling chamber with circulating water. Thermal effects were minimized by keeping samples at (24±1)℃ with circulating water. In the still experiment, a sterile 5 mL or 10 mL polypropylene centrifuge tube was used to carry out UE. In the experiment of flowing state, UE was performed by the flow of red blood cell suspension through a rubber tube (ø 2 mm) of infusion pump in water tank, simulating the speed of blood flow. The base of tank was sealed and filled with filtered deionised water. The sample tube was placed within the ultrasonic exposure tank, and exposed to US at room temperature (24±1)℃ using a combination of 2-5 intensities (0 to 1 W/cm^2), 10 total US exposure times (TUET, 1 to 24 minutes). The tubes were positioned directly in front of the transducer facing the tube axis at 4 cm from the transducer (hydrophone was at the same position), and the surrounding water in the tank was kept at (24±1)℃ to minimize the thermal effects on cells.

Evaluation of Delivery Parameters

The possible mechanism of ultrasonic naked gene delivery is based on increasing the permeability of cell membrane and creating instantly-recovered porosity on cell membrane caused by cavitation[9]. Therefore, the suitable ultrasound intensity (UI), total ultrasound exposure time (TUET), the threshold of cell trauma and cell viability are important factors[14], which needed to be determined before naked gene delivery.

In the still experiment, the red blood cell sample of rabbits, rats, and chickens was respectively exposed to US field with different TUET and UI. After exposure, the cell viability was evaluated by cell counting and the morphology was observed by microscopy (Leica DM RXA, Germany). The porous prodding red blood cells and cell viability were the objects of evaluation. They allowed estimation of the degree of damage to the cell membrane, and by combination with hemoglobin analysis, they could help to determine the optimal permeable parameters of the cell membrane. The supernatant of red blood cells was collected by centrifugation (Boifuge 15R , HEERAEUS German; 200 g, 5 min, 4℃). The absorbance of haemoglobin in the supernatant was determined at 575 nm using a spectrophotometer (DU-64, Beckman) with a standard method[15]. The value of haemoglobin in the supernatant indicated the degree of membrane permeability. The control group without UE was evaluated in the same way.

In the experiment of flowing state, a wheel infusion pump (WSQ-A, Wheel Infusion Pump, Jiangsu, China) was used to create an environment. Twenty mL red blood cell suspension of rats was added into the pump to circulate through a 2 mm rubber tube placed at the same spot as the tube exposed. After exposed to different TUET and UI, the supernatant of red blood cells was collected by centrifugation. The optic value of haemoglobin in the supernatant was determined by a standard method[15].

To validate the threshold of cell trauma and viability, the morphology of S180 cells was estimated immediately after UE with microscopy and scanning electron microscopy (SEM, JSM-5600, Japan) by addition of 10 μL of 0.01% trypan blue. We also evaluated the viability and damage of S180 cells by flow cytometry (FCM, Epxcs XL Coulter, USA) using the propidium iodide dyeing (PI)[16], confocal laser scanning microscopy (CLSM), as well as the Acrine Orange (AO) method. According to the analysis of morphology, enzymes, free radicals and value of haemoglobin in the supernatant, optimal UI and TUET were determined. The control group without UE was tested in the same manner. The cell morphology was observed at 1 to 24 minutes of TUET. To assess the cell function, the exposed S180 cells were re-inoculated into the abdomen of small white mice. Four days later, ascites was collected by abdominocentesis and washed 4 times with PBS and examined.

Reporter Gene

The plasmid vector containing *pcDNA3.1/CT-GFP* (kindly provided by Dr. Yang G.R, Fourth Military Medical University of China), was used as a reporter gene and amplified in *E. coli*, extracted and purified with plasmid miniprep kit (Omega, USA). It carried the green fluorescent protein (GFP, 488 nm) under the transcriptional control of the strong constitutive cytomegalovirus (CMV) promoter and was possibly in live cells without any further processing. The purity of plasmid was examined by UV spectroscopy (UV-265FW, SHIMADU CORP, JAPAN; E260/E280 nm ratios ranging from 1.87 to 1.89 were used) and electrophoresis. The adeno-associated virus vector containing *GFP* (*AAV-GFP,* AGTC Gene Technology, China) was diluted to 10^4 v.g/mL for comparison with the transfection of naked plasmid *GFP* delivery with UE. The transfection was carried out according to the manufacturer's instructions.

Gene Delivery and Expression

After having determined the optimal parameters of UE, we utilized the naked plasmid *GFP* to evaluate naked gene delivery efficiency. The sample was divided into four identical groups: A, B, C, and D. Group A served as the test group, 0.1 μg/mL naked plasmid *GFP* gene was added into every tube with UE. Group B was the comparison group, 10 μL *AVV-GFP* vector 10^4 was added without UE. Group C served as the control group, 0.1μg/mL naked plasmid *pcDNA3.1/CT-GFP* gene was added without UE. Group D served as the blank group without UE and *GFP* gene. After UE, 5% HFBS was added into every tube and cultured as suspension in a humidified atmosphere of 95% air and 5% CO_2 at 37℃ for 48 h to evaluate UNGD efficiency. The fluorescence microscopy (FM, Leica DM RXA, Germany) and confocal laser scanning microscopy (CLSM, True confocal scanner, Leica TCS SPII, Germany) were used to observe UNGD efficiency and gene expression, and images were photographed with CCD camera MPS60. Histogram analysis was used to evaluate the intensity of *GFP* expression.

Enzymology and Free Radical

The S180 cell suspension was exposed to US with different TUET and UI. Acid phosphatase (ACP,

520 nm), alkaline phosphatase (AKP, 520 nm), malondialdehyde (MDA, 532 nm), hydroxyl free radical (OH, 550 nm) and superoxide dismutase (SOD, 550 nm) were detected by spectrophotometry (ACP, AKP, MDA, OH and SOD detection kits, Jiancheng Bio., NJ, China) using a spectrophotometer (DU-64, Beckman, Thermostat Water Bath Tank) according to the manufacturer's instructions.

Statistical Analysis

SPSS 10.0 and CurveExpert 1.3 were applied in data analysis. The results were expressed as mean±standard error of the mean (SE). Statistical analysis of results was performed using the Student's *t*-test to assess significance of the test samples in comparison with that of the control groups. $P<0.05$ was considered statistically significant.

RESULTS

Assessment of Bioeffects Parameters

Ultrasound intensity (UI) and total ultrasound exposure time (TUET) at 90% cell viability were determined as the observational points and optimal parameters for UNGD. The porous, acanthoid and abnormal red blood cells were considered as the objects of evaluation. Figures 1 and 2 show the results from the rabbit red blood cells with 0.7 W/cm^2 UI, in which (90±1.41)% (n=6) cell viability was accompanied with (80.17±2.04) % (n=6) porous cells and (89±1.41)% (n=6) acanthoid cells at 5 minutes of TUET. The porous and acanthoid cell membrane began to recover 15 minutes after UE. Another result of rabbit red blood cells with UE at 0.5 W/cm^2 UI showed that (89.83±1.72)% (n=6) cell viability was accompanied with (80±1.79)% (n=6) porous cells and (90±1.41)% (n=6) acanthoid cells at 7 minutes of TUET. To validate the results above, chicken red blood cells were respectively exposed to different UI at 0.7, 0.6, 0.5, 0.4, and 0.3 W/cm^2. The results of the cell morphology revealed that 5 minutes of TUET with 0.7 W/cm^2, 6 minutes of TUET with 0.6 W/cm^2, 7 minutes of TUET with 0.5 W/cm^2, 9 minutes of TUET with 0.4 W/cm^2 and 12 minutes of TUET with 0.3 W/cm^2 were near to an optimal parameter. Findings from chicken red blood cells revealed (89.8±0.84)% (n=5) cell viability and (80.2±1.92)% (n=5) abnormal cells. Figure 3 shows the results from 0.6 W/cm^2 UI. Other tests of S180 tumour cells also showed the similar results to those with rabbit red blood cells and chicken red blood cells at 5 minutes of TUET with 0.7 W/cm^2 UI (Figure 4, $P>0.05$, *t*-test). The cell viability at 5 minutes of TUET was (90.8±1.64)% (n=5). Combined analysis of all the figures showed that, as TUET and UI increased, cell viability generally decreased and the number of porous, abnormal and acanthoid cells increased. By fixing monitoring point at 90% cell viability, we found that as UI increased, TUET decreased. The energy flux of UE seemed to approach a constant E, which had a relationship with UI, cell membrane permeability and TUET. At 90% cell viability, we supposed that the constant E was the optimal energy parameter of UNGD, E=TUET·UI. With different TUET and UI, the results from different cell types were very similar ($P>0.05$ by *t*-test) and supported each other.

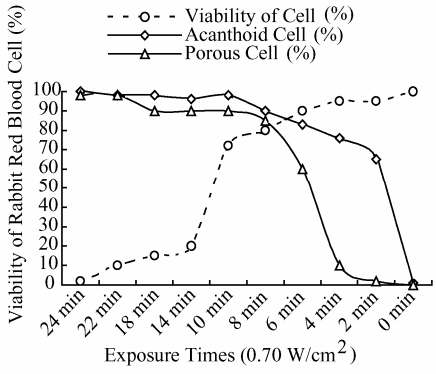

FIG.1. Bioeffect evaluation of rabbit red blood cell at 0.7 W/cm^2.

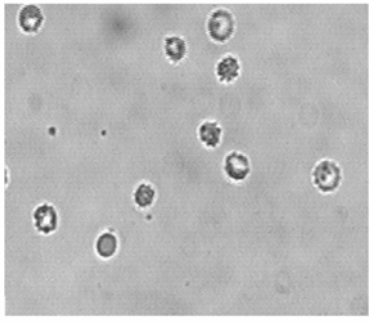

FIG. 2. Porous and acanthoid red blood cells of rabbit at 0.7 W/cm^2 40×40.

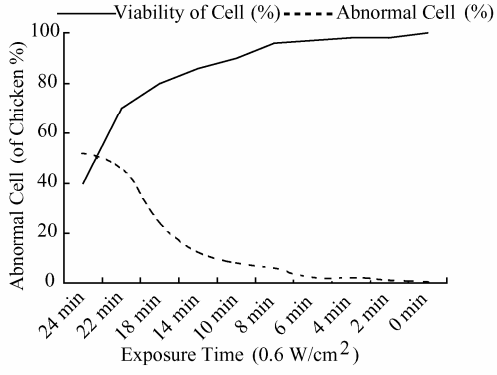

FIG. 3. Results of chicken red blood cells at 0.6 w/cm^2.

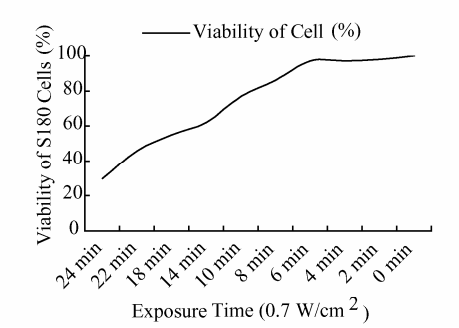

FIG. 4. Viability of S180 cells at 0.7 W/cm^2.

Another test was used to prove the safety of the optimal energy parameters. We assayed the haemoglobin in red blood cell supernatant of rats after UE. The optimal parameters of 5 minutes of TUET at 0.7 W/cm^2 UI were validated by the change of permeability of haemoglobin through rat red blood cell membrane. The increase of haemoglobin in the supernatant indicated the rise of permeability of cell membrane. The results showed that the optic density (OD) of haemoglobin in the supernatant began to rise rapidly at 5 minutes of TUET with 0.7 W/cm^2 UI. We also observed the change of haemoglobin in the supernatant of rabbit red blood cells, simulating a speed of blood flow. Fig. 5 shows the results from different UI and TUET in the flowing state. They proved again that 90% cell viability and energy accumulation at this point were optimal parameters for bioeffects of cell membrane permeability and UNGD. We could use it to deliver naked gene into cells. The results above were consistent with morphological observations. It was proved that optimal parameters from morphological observation were valid. UI and TUET had a positive correlation with the permeability of cell membrane. UI showed a negative correlation with TUET. The energy parameter E of UNGD had the same relationship with UI and TUET as described above. Besides the optimal parameter point, there was another sharp increase of haemoglobin at 7 minutes of TUET, which nearly corresponded to 80% cell viability. This could be determined as the threshold of cell damage and E should be limited to 5.97±0.55 (n=6).

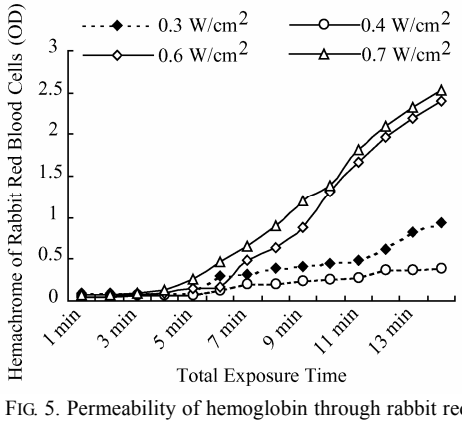

FIG. 5. Permeability of hemoglobin through rabbit red blood cell membrane.

Bioeffects of Gene Delivery and Expression

We used the optimal parameters of 5 minutes of TUET at 0.7 W/cm^2 to deliver naked plasmid *GFP* gene into S180 cells. As mentioned above, the experiment was divided into four groups: A, B, C, and D. Forty-eight hours after UE, we obtained the results by CLSM (Fig. 6) and FM (Fig. 7). In group A, naked plasmid *GFP* gene was delivered into S180 cells, showing a higher fluorescent intensity than in group B. The expression in group C was very weak. Histograms of group A showed three high-fluorescence peaks with a low-fluorescence background, indicating a large amount of naked plasmid *GFP* uptake within each population of cells. Fig. 8 shows histogram analysis of expression intensity. In group B, it showed broad distribution of low-fluorescence background without a peak, suggesting a small amount of *GFP* uptake within each population of cells and a high transfection rate. Although the cell viability with *AVV-GFP* vector was higher than that with ultrasound method ($P<0.05$ by t-test), approaching (98.17±0.75)% (n=6), histogram analysis proved that the intensity of *GFP* expression yielded by UNGD was obviously higher than that by *AVV-GFP* vector 10^4 (group B, Figures 7, 8, $P<0.001$, t-test) and plasmid *GFP* gene without UE (group C, Figures 7 and 8, $P<0.001$, t-test). To estimate the transfection rate we counted *GFP* positive and negative cells in the FM. The transfection rate was (40±2.2)% (n=6) for *AVV-GFP* vector and (35.83±2.53)% (n=6) for UNGD in viable cells, with (90.17±1.47)% (n=6) of cell viability. In Fig. 8, the low-fluorescence background indicates transfection rate, and the high-fluorescence peak stands for expression intensity. The results agreed with the counting results of positive and negative cells.

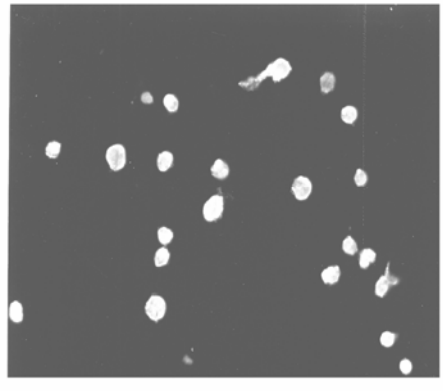

FIG. 6. *GFP* delivery results and expression examined by CLSM after ultrasound gene delivery.

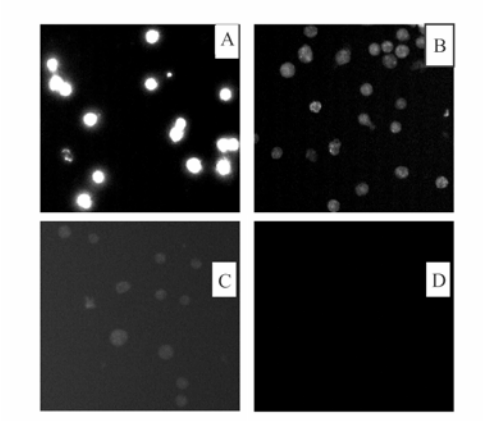

FIG. 7. *GFP* delivery results and expression, checked by FM. A: test group with high fluorescence, gene delivered by ultrasound at 5 min with 0.7 W/cm^2. B: *AVV_GFP* group with low fluorescence. C: control group with weak expression. D: Blank group without UE and *GFP*.

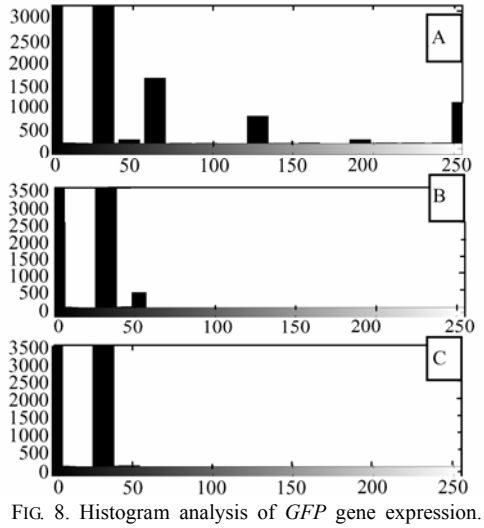

FIG. 8. Histogram analysis of *GFP* gene expression. A, B, C: groups A, B, and C.

Safety Evaluation After Gene Delivery

To estimate cell trauma after UNGD, we conducted SEM (×5000, 20kV), CLSM and FCM. There were no traces of cell membrane destruction at 3 minutes of TUET or damage peaks in FCM histogram (Fig. 9A). At 5 minutes of TUET, a trace of recovered cell membrane destruction (Fig. 9B) was found with 2.1% damage peak in FCM histogram. At 8 minutes of TUET, pores did not recover well with a 5.3% damage peak in FCM histogram (Fig. 9C). The findings from LSCM at 8 minutes of TUET indicated pyknotic nuclei in cells (Fig. 10A) and karyostenosis (Fig. 10B), which were the signals of nuclear trauma. At the same time the exposed S180 cells were re-ino culated into the small white mouse abdomen. After four days, the S180 cells grew well and (24.1±1.2)% (n=5) showed *GFP* expression.

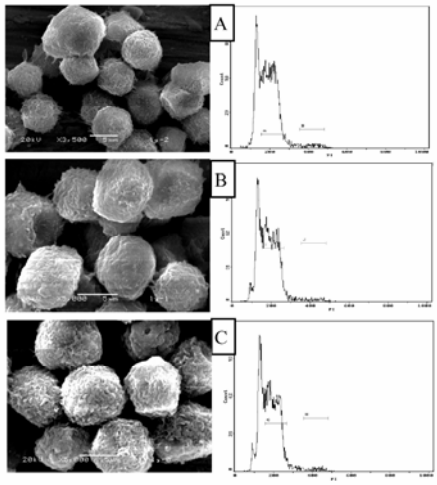

FIG. 9. Evaluation of cell trauma with 0.7 W/cm^2. Right was results of SEM. Left was results from FCM. A: cells exposed for 3 min without evident damage. B: cells exposed for 5 min with trace of recovered pore. C: cells exposed for 8 min with uncovered holes and 5.3% damage peak.

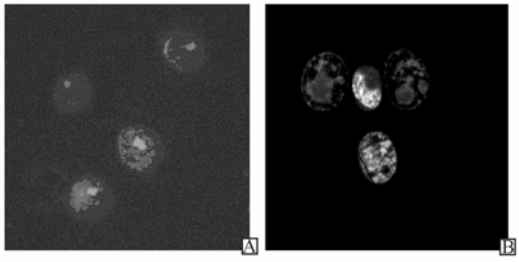

FIG. 10. Nucleolus damage beyond threshold at 80% of cell viability with AO fluorescence checked by LSCM. A: pyknotic nuclei B: karyostenosis indicating damage of cell.

After UNGD with UE at 0.7 W/cm^2 UI, malondialdehyde (MDA), superoxide dismutase (SOD), hydroxyl free radical (OH$^{\bullet}$), alkaline phosphatase (AKP), and acid phosphatase (ACP) were detected. MDA increased with the elongation of TUET (2.8±0.2 to 7.38±0.14, n=5 at about 8 minutes). When it began to increase rapidly, it responded to damage threshold of cell membrane. SOD showed a decreasing tendency with increase of exposure energy accumulation (68.71±3.1 to 10.26±2.3, n=5, Fig. 11). OH$^{\bullet}$ had an increasing tendency (299.93±7.43 to 422±6.82, n=5, Fig. 12) which was consistent with change of MDA and SOD. AKP (33.75±0.42 to 34.79±0.63, n=5) and ACP (4.97±0.53 to 8.92±0.76, n=5) also tended to elevate with increase of energy accumulation. Except for SOD, most results showed the tendency of sigmoidal or S-shaped growth model, $y=(ab+cx^d)/(b+x^d)$, where y indicates the amount of

MDA, SOD, AKP, and ACP; *x* is the TUET, and *a, b, c,* and *d* are the coefficients of S-shaped growth model.

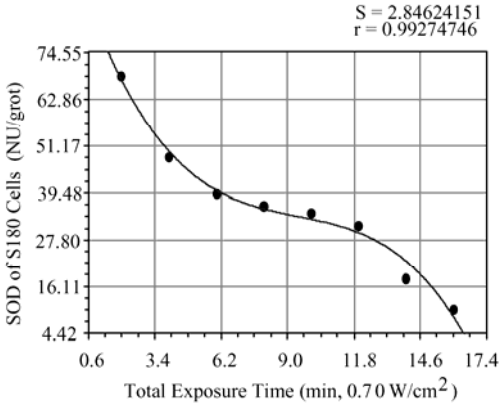

FIG. 11. Decrease of SOD with increase of ultrasonic exposure time.

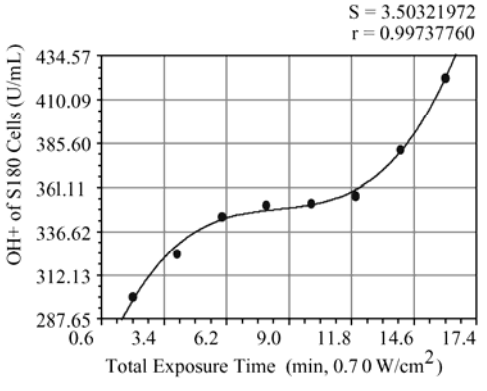

FIG. 12. OH+ showing a rising tendency with increase of ultrasonic exposure time.

DISCUSSION

Genetic diseases can be cured by proper replacement and manipulation of genes. Numerous attempts have been made to overcome the relatively low transfection efficiency of naked DNA gene transfer, including use of cationic lipids or polymers[18-20], or application of electric pluses to DNA injection sites[17]. We reported here an ultrasound method for efficiently delivering naked gene into cells. US can penetrate a number of materials such as biological soft tissues and has many biological effects, thus it can be applied to disease treatment. It also provides a non-invasive method for cancer gene therapy. To understand the mechanism of gene delivery and to explore the potential application in cancer gene therapy, the influence of different UE and cell conditions on UNGD efficiency was studied here. Our findings indicate that maximizing gene delivery efficiency depends on increasing membrane porosity while avoiding irreversible cellular damage. US increases the permeability of cell membrane to enhance an uptake of naked *GFP* gene under optimal conditions. The results suggest that US might be more likely to induce the optimal balance between membrane pore formation and recovery in cells. We monitored the safety of UNGD by microscopy, LSCM, SEM, and spectrophotometry, and found that the parameters at 90% cell viability were optimal for delivery of naked gene into cells without irreversible cell damage. The significant observation from this study is that the permeability of cell membrane in response to US shows a nonlinear pattern of energy accumulation. At 90% cell viability in different cells, we found that the energy accumulation of UE seemed to approach an energy flux constant *E*, which is more useful in controlling bioeffects combined with the measurement of one-half of the applied frequency[2]. When energy accumulated to the point of 90% cell viability, the permeability of cell membrane and free radicals began to increase the uptake of naked DNA. When energy reached a point of 80% cell viability, the cell membrane showed burst permeability with cell damage, a sharp rise of free radicals and AKP, ACP. It was illuminated that 80% cell viability was the damage threshold of cell membrane. This study showed that permeability increase was a strong effect of UE and TUET, indicating that the selection of appropriate UI and TUET is important to achieve permeability of cell membrane at an optimal level. With the increase of TUET and UI, MDA, AKP, ACP, and $OH^{\bullet}$ free radicals showed the upward tendency, and SOD decreased at the same time. This fact proves that the ultrasonic bioeffects of enhancing cell membrane permeability are related to the amount of $OH^{\bullet}$ free radicals. Thus, the free radical is one of the main reasons why ultrasound increases the permeability of cell membrane to deliver gene into cells. Below damage threshold, MDA, AKP, ACP, and $OH^{\bullet}$ free radicals showed little increase.It is proved that the energy accumulation at 90% cell viability is valid.

The success of gene therapy has been limited by the inability of genes to cross biological barriers and cell membrane in the body[16]. Several methods have been developed for delivery of DNA into cells, including chemically-facilitated, vector-mediated, mechanical[17], ballistic technology and electric pluses methods[14]. In the current clinical protocols of gene therapy, virus-derived vectors and non-virus-derived vectors have been used in most trials[7]. Each method has its inherent limitations, such as low efficiency, complex protocol, and high cost. Moreover, many cells are only responsive to certain methods. Virus-derived vectors have transfection

efficiency superior to non-viral vectors. In spite of this advantage, they involve immunogenicity, cytotoxicity, possibility of mutagenesis and have some technological difficulties and high cost[8]. One promising approach to naked gene delivery is to use UE to instantly open cell membrane to deliver genes into cells, which has been proved in our study. In this respect, UNGD method is a more versatile and promising technique, because it is based on the modification of cell membrane permeability and is less dependent on the type of cells and DNA. As a physical method, UNGD can be applied to different cells without modifying the structure of genes[3,5-6], and it is readily available because of its ability to focus on any part of the body to deliver genes into cells. In addition, it does not involve immunogenicity, cytotoxicity, and possibility of mutagenesis. It is rapid, relatively inexpensive, and easy to be located in deep tissues of the body for gene therapy. The experimental results *in vivo* will be reported elsewhere.

The cavitation is regarded as the mechanism responsible for ultrasonic penetration of cells and gene delivery. It involves the production and oscillation of gas bubbles in a liquid. In the low-pressure portion of ultrasound wave, dissolved gas and vaporized liquid can form gas bubbles. Then, the bubbles shrink and grow in size, oscillating in response to the subsequent high and low pressure portions of the ultrasound wave. This is called stable cavitation. Another type of cavitation is referred to as transient cavitation, which occurs under greater acoustic pressure, where bubbles violently implode after a few cycles[13]. The procedure of implosion can produce many effects, including a transient rise in local temperature and local pressure, generating instant free radicals, and launching a high velocity liquid microjet. In our study, the results might have a relation with stable cavitation, yielding optimal parameters of gene delivery with little rise of MDA, AKP, ACP, and OH+ free radicals. On the other hand, the increase of $OH^{\bullet}$ free radicals might cause the apoptosis of tumour cells (Figs. 10-12). Based on this fact, UNGD can be used to treat cancer directly with suitable UE and UI.

CONCLUSION

The results suggest that both ultrasonic cavitation and free radicals are responsible for bioeffects of UNGD. The advantages of UNGD are that the efficiency is not significantly influenced by the stage of cell growth and transfection can be done in complete medium with serum. These advantages along with the ability to focus ultrasound in most areas of the body provide a novel approach to medical treatment modalities such as cancer gene therapy. Though previous studies have achieved enhanced effects in gene transfection[21-22], low frequency US can safely deliver naked gene into cells without damage of cell function under optimal conditions. *E* as a control factor, can be applied to controlling bioeffects combined with other parameters. The MDA, AKP, ACP, and OH+ free radicals show a positive correlation to the permeability of cell membrane, while SOD shows a negative correlation. This study may be helpful for development of a novel clinical gene therapeutic system, especially for cancer therapy.

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