Different Effects of Therapeutic Ultrasound Parameters and Culture Conditions on Gene Transfection Efficiency

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ABSTRACT

Objective: To investigate the effect of different therapeutic ultrasound (TUS) parameters and culture conditions on the cell viability and transfection efficiency of human cervical cancer cells (HeLa). **Methods:** HeLa cells were cultured using two different protocols (in suspension or in monolayer). Subsequently, cells were exposed to different TUS intensity (0.4 W/cm^2, 1.0 W/cm^2, 1.6 W/cm^2, 2.2 W/cm^2), duty cycle (DC)(10%, 20%, 50%), exposure time (1 min or 3 min). Cell viability was analyzed by flow cytometry. Gene transfection of red fluorescent protein (DsRED) was detected. **Results:** TUS intensity and duty cycle had a great impact on the overall results (P<0.01). Cell injury were found to increase progressively with intensity (1.6 W/cm^2, 2.2 W/cm^2) and duty cycle (50%) and cell detachment was accompanied by ultrasound exposure in adherent cells. Results of factorial design showed that the fashion of cell culture and the TUS parameters had interaction (P<0.01). The ideal conditions that cell viability above 80% producing maximum efficiency were noted to be at 1.0 W/cm^2 irradiated 3 min with a duty cycle of 20% in cell suspension. **Conclusion:** TUS parameters and transfection conditions have a great impact on the gene transfection and cell viability. Optimal parameters could enhance cell membrane permeability, which facilitate to delivering the macromolecules into cells.

Key words: Therapeutic ultrasound; Gene therapy; transfection; Cavitation; Cell viability

Therapeutic ultrasound (TUS) that is currently used for physical therapy in clinical fields can thereby increase drugs or gene transfer[1,2]. TUS, which operates at moderate frequencies of 1-3 MHz and intensities of 1-3 W/cm^2, is being utilized widely[3]. It has good penetration through soft tissues, does not damage cells and tissue or affect integrity of DNA. TUS is superiority to low- frequency ultrasound or high-intensity focused ultrasound[3,4]. Using TUS to deliver specific drugs or genes into cells for treatment, or induce the biological effects with a certain treatment effect, is an investigation field developed rapidly[4-6]. But further modifications of the relationship between TUS parameters and culture conditions are necessary to achieve better efficacy of the TUS mediated drug or gene therapy or transfer. For this reason, this study was designed to investigate the effects of different culture ways and TUS parameters, with the purpose of determining the optimal parameters combination, expecting to achieve the best parameters and transfection protocol with minimum cell damage, and laid the foundation for non-invasive drug or gene therapy.

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MATERIALS AND METHODS

Cell Culture

HeLa cells were obtained from China Center for Type Culture Collection (CCTCC) and incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/ml penicillin, 100 μg/ml streptomycin, at 37°C in a humidified environment of 5% CO_2/ 95% air. Total cell count was determined with a hemocytometer (Burker Turk). Initial cell viability was determined by means of exclusion with trypan

blue dye (Sigma-Aldrich, USA). Exponentially growing cells were used in all experiments.

Plasmid DNA

The plasmid pDsRED-Express (6.7 kb), an expression vector for red fluorescent protein (DsRED), whose excitation and emission maxima occur at 557 nm and 579 nm, respectively, was obtained from BD Biosciences Clontech, USA. The plasmid DNA obtained from DH5α Escherichia *coli* (Invitrogen, Carlsbad, CA, USA) was prepared with a Kit (Qiagen, Crawley, UK) according to the company protocol. The absorbance ratio at the wavelength of 260–280 nm for the plasmid DNA solution was measured to be between 1.8 and 2.0.

Experimental Apparatus

All operations were performed in the sterile decontamination bench. All *in vitro* experiments were performed in an exposure water-tank. The tank contained deionized water with the ultrasound transducer (Accusonic, Metron Medical Australia Pty. Ltd.) with frequency of 1 MHz, pulse repetition frequency of 100 Hz, fixed at the bottom of it. The culture plate was placed above the center of the transducer and covered with the lid to prevent from being polluted by surrounding environment. To prevent the nearby well being affected by ultrasound irradiation, cells were planted in only six wells of 24-well culture plates. During TUS irradiation, the plate was moved slowly as circumference, sticking closely to the transducer surface.

Experimental Design

To study the effects of TUS and cell detachment on cell injury, the differences of survival rate were compared under different TUS parameters and the culture conditions, so as to determine the combination of optimal parameters in the subsequent experiments of transfection protocol. We used the four factors factorial design. The conditions of the cultured cells were divided into two levels at adherent sample (Group A) and suspended sample (Group S). TUS intensity was divided into four levels, i.e., 0.4 W/cm^2, 1.0 W/cm^2, 1.6 W/cm^2 and 2.2 W/cm^2. While the duty cycle (DC) was divided into 3 levels, i.e., 10% (1 ms on，9 ms off), 20% (2 ms on，8 ms off) and 50% (5 ms on，5ms off). The exposure time was divided into two levels of 1 min and 3min.

Group A: One day before experiments, cells were trypsinized and plated to reach 80%-90% confluence level in 24-well plates to a concentration of 1×10^6/ml with 300 μl non-FBS culture media per well. SonoVue microbubble (100 μl/well，Bracco) were added to the medium before exposure. 4 h after TUS irradiation, cells were washed with PBS. Then exchanged by DMEM medium containing 10% FBS to remove non-viable cells and cell debris.

Group S: Before irradiation, cells were harvested by trypsin treatment, washed twice in PBS and collected, with the same density as group A as described above, then added into the cultivate plate with microbubbles. The suspension was exposed to TUS immediately at room temperature, and hold up for a proper time which was not enough for cells to adherence. The medium was replaced 6 h after TUS irradiation to continue to cultivate.

Cell Viability and Transfection Efficiency

Group A was incubated at 37^{o}C for 24 h, while group S for 48 h, the DsRED expression was observed with fluorescence contrast phase microscope (IX71, Olympus, Japan). Then the cells were harvested, collected, centrifuged and resuspended in PBS buffer. 20 μl of propidium iodide (PI, 40μg/ml, Sigma-Aldrich, USA) was added into the cell suspension. Cells with PI were considered as dead and without PI as alive. After being incubated at room temperature for 15 min, the cells were stored at 4^{o}C. Using flow cytometry (FACS Calibur, Becton Dickinson, USA), ratios of cells stained with or without PI were determined.

About 10^5 cells were taken from each sample for transfection efficiency analysis, with 488 nm wavelength excitation light, (585±42) nm wavelength emission light to detect the red fluorescent. The gene transfection efficiency was assessed as the number of cells expressing DsRED per total number of survival cells. Data was analyzed using Cell Quest Pro software.

Statistical Analysis

Statistical analyses were performed by the SPSS 13.0 software package (SPSS, Inc, Chicago, IL). All values were expressed as $\bar{x}\pm s$. Analysis of variance with paired *t* test and analysis of variance (ANOVA) of factorial design were used to determine the significance of the difference in a multiple comparison. If the ANOVA was significant, the Tukey's and Dunnett-*t* procedure were used as a post hoc test. Analysis of variance of factorial design was also used to analyze the main effect and interaction between culture conditions and TUS parameters. Differences with a *P* value of less than 0.05 were considered to be statistically significant