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利用超音波來進行體外基因轉移之研究

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中文摘要

如何發展安全及有效的基因傳送方法在基因治療中一直是主要的挑戰，本研究主旨在於找出最佳化的超音波聲參數使基因轉移效率以及細胞存活率皆能增加，我們有興趣的參數包括超音波脈衝重複頻率(pulse repetition frequency)、暫停時間(off time)以及時間比率對體外培養細胞的影響。本研究中發現，對於子宮頸癌細胞(HeLa cell)而言，當超音波工作週期(duty cycle)為 50%時，最佳脈衝重複頻率為 20 Hz 到 1000 Hz 之間；當脈衝重複頻率低於 2 Hz 時，細胞擁有高轉移效率，但會造成高死亡率及高溫升；當脈衝重複頻率高於 1000 Hz 時，轉移效率明顯開始下降，而當脈衝重複頻率高於 4000 Hz 時，細胞雖然有高存活率及低溫升，但轉移效率卻為 0%。另外由實驗結果中可發現，超音波每一次的施打時間與暫停時間比例(on-time/off-time)需大於 0.1，當此比例小於 0.1 時，轉移效率急速下降。

關鍵詞：超音波、基因治療、基因轉移

英文摘要

It is always a great challenge to develop a safe and effective method for delivering gene into cells. The main purpose of this study is to determine optimal acoustic parameters for effective gene transfer and high cell survival in the ultrasound-mediated gene transfection. The parameters we studied included the pulse repetition frequency (PRF), the length off time and the ratio of on to off time (on-off ratio). We found that for cultured cervical cancer cells (HeLa cells), the best PRF for gene transfection ranges between 2 and 2000 Hz (50% duty cycle). The transfection efficiency declined rapidly while PRF was higher than 1000Hz and dropped to 1% while PRF was 4000 Hz. When the ultrasound pulse repetition frequency (PRF) was shorter than 2 Hz (the exposure on-time was larger than 250-ms), the transfection efficiency was high, but the extremely high temperature elevation resulted in low cell survival. While PRF was between 20 Hz to 1000 Hz, a better transfection could be obtained when the ratio of the on-time to the off time was larger than 0.1, i.e. $\frac{on-time}{off-time} > 0.1$. When the ratio was smaller than 0.1, the transfection efficiency was close to 0%.

Keyword: ultrasound, gene therapy, gene transfer, gene transfection

I. 前言與研究目的

Recently, gene therapy becomes increasingly important for the treatment of inherited or acquired disorders, such as atherosclerosis and cancer. However, results of clinical studies have not always been satisfactory. One of the main difficulties is the methods of the gene delivery. In principle, there are two major classes of vehicles for gene transfer: viral and nonviral vectors. Nonviral vectors may circumvent some of the problems occurring with viral vectors, such as endogeneous virus recombination, oncogenic effects and unexpected immune response. Several nonviral techniques are under development, including ultrasound. Ultrasonic waves are non-ionizing mechanical waves and can be focused to a target region for localized gene transfection. It is believed that ultrasonic mechanical waves or other subsequent interaction mechanisms can alter the cell membranes permeability and might thus be able to allow macromolecules, such as plasmid DNA, to move into cells.

Although the ability of ultrasound for enhancing gene transfer has been demonstrated in numerous studies, the mechanism by which transiently permeabilizes cell membranes remains poorly understood. It is generally believed that ultrasound-mediated bioeffects on cell membranes are related to acoustic cavitation, instead of ultrasonic thermal effect. Cavitation may make some “temporal holes” on cell membranes and facilitate gene transport. It could also make irreversible damage and lead to cell death.

The ultrasound biological effects, including transfection abilities and cell death, depend highly on parameters such as acoustic parameters. The aim of this study is to find out a set of optimal parameters for better gene transfer but less cell death, especially the effects of on-off time duration and pulse repetition frequency (PRF).

II. 文獻探討

A study of cavitation activity induced in water by exposure to ultrasound of variable pulse length revealed interesting results. Ciaravino et al. (1981) showed that inertial cavitation activity determined by the iodine release method had a maximum at pulse durations of ~6 and 60 ms for a 1MHz exposures at 0.94 and 0.77 MPa, respectively. The total ‘on’ time was fixed at 60 s with constant 50% duty cycle, and pulse durations varied from 60 μs to 60 s. In a subsequent study using whole blood and Albunex, ultrasound-induced hemolysis increased generally with increasing pulse durations, but a

reproducible local maximum was found for pulse durations of 20 to 30 μs (1MHz) at intensities on the order of 400 to 800 W/cm^2 (Brayman et al. 1996).

A possible mechanism has been proposed to explain the pulse duration dependence of inertial cavitation activity. Flynn and Church (1984) suggested that unstabilized nuclei surviving from one inertial cavitation event to the next brought about the observed maxima in inertial cavitaiton activity at intensities greater than the threshold value, and at pulse durations of 6 and 60ms. A recent study by Chen et al. shows that with constant acoustic energy delivered, ultraound exposures with longer pulse length or high PRF generated more inertial dose and hemolysis. The explosive increase of generated bubbles (‘cascade’ effect) occurred at long pulse lengths or high PRF conditions. Under these conditions more bubbles survived through the ‘off’ period and helped generate more inertial cavitation activity and hemolysis with subsequent high-intensity pulses (Chen et al. 2003).

III. 研究方法

Cervical cancer cells (HeLa) were routinely cultured as a monolayer in culture flasks covered with Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and 1% Antibiotic-Antimycotic (penicillin G, streptomycin, and amphotericin B ,GIBCO,USA)in a 37°C humidified incubator (5% CO_2). One day before the ultrasound exposure, the cultured cells were trypsinized and seeded at a concentration of 3.5x10^4 cells in a well of 24-well plates (Corning Incorporation, NY, USA). Each plate was seeded six wells. Right before the ultrasound exposure, the cultured cells were immersed in 0.5ml DMEM solution mixed with plasmid.

The enhanced green-fluorescent protein (EGFP) reporter gene was used in our studies mainly because of its high sensitivity and convenience in quantification of gene expression. The pEGFP-C1 plasmid encodes a variant of the Aequorea Victoria green fluorescent protein (GFP) that has been optimized for brighter fluorescence and higher expression in mammalian cells. The half life of EGFP is 24 hours. Plasmid size is 4.7 Kb and molecular weight is 27 kD. The pEGFP-C1 plasmid was purchased from BD biosciences. Plasmids amplified by E.coli cultures and purified by Giga kit (BD biosciences, San Jose, CA, USA). The EGFP plasmid were added to the DMEM solution and mixed gently before the ultrasound exposure. Then the cultured cells were immersed in 0.5 ml DMEM solution mixed with plasmids right before the ultrasound exposure.

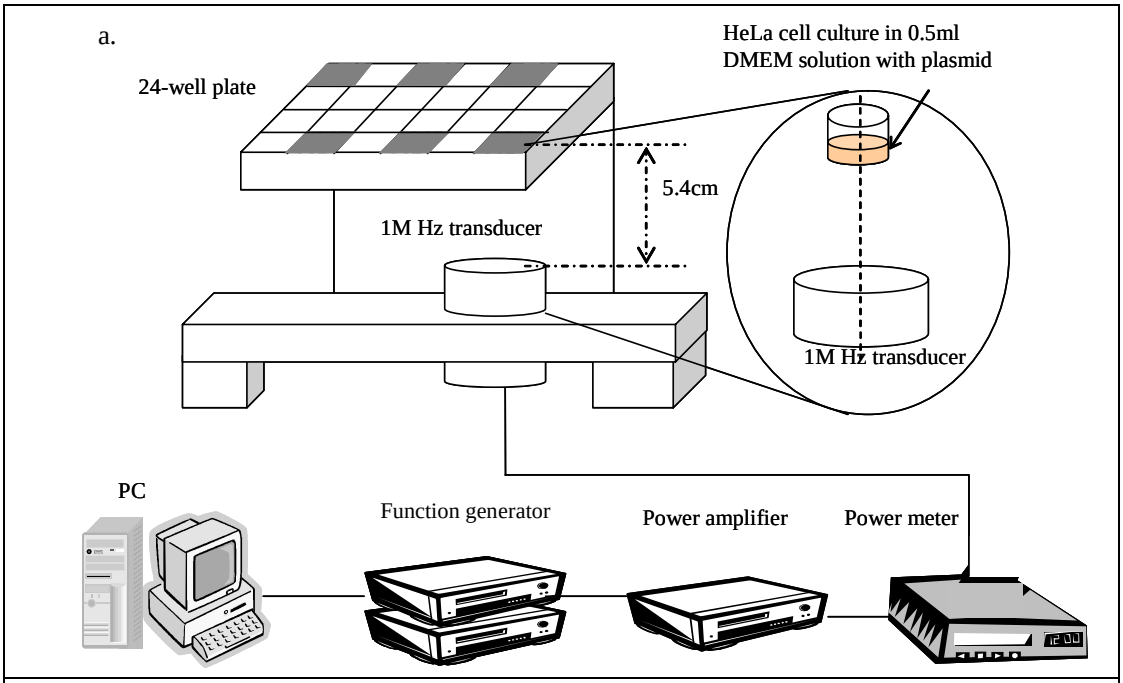

Fig. 1: Experimental setup for the ultrasound-mediated gene transfection

All experiments were performed in a tank containing 37 ℃~ 35 ℃ water. The ultrasound field was generated with a 1-MHz plane air-backed piezoelectric transducer with 3.8 cm in diameter and its focal length is 24.3 cm (A392S, Panametrics, Waltham, MA, USA). A 24-well plate was placed 5.4 cm above the transducer (Fig. 1). One well was exposed by ultrasound each time. The center of the exposed well was aligned to the center of transmitting transducer.

For the actual experiments, several combinations of PRF and on-off ration were tested. The duty cycles were all 50%. All the total ultrasound exposure times were 10-sec and the ultrasound intensity was set to 4.8 W/cm^2.

IV. 結果

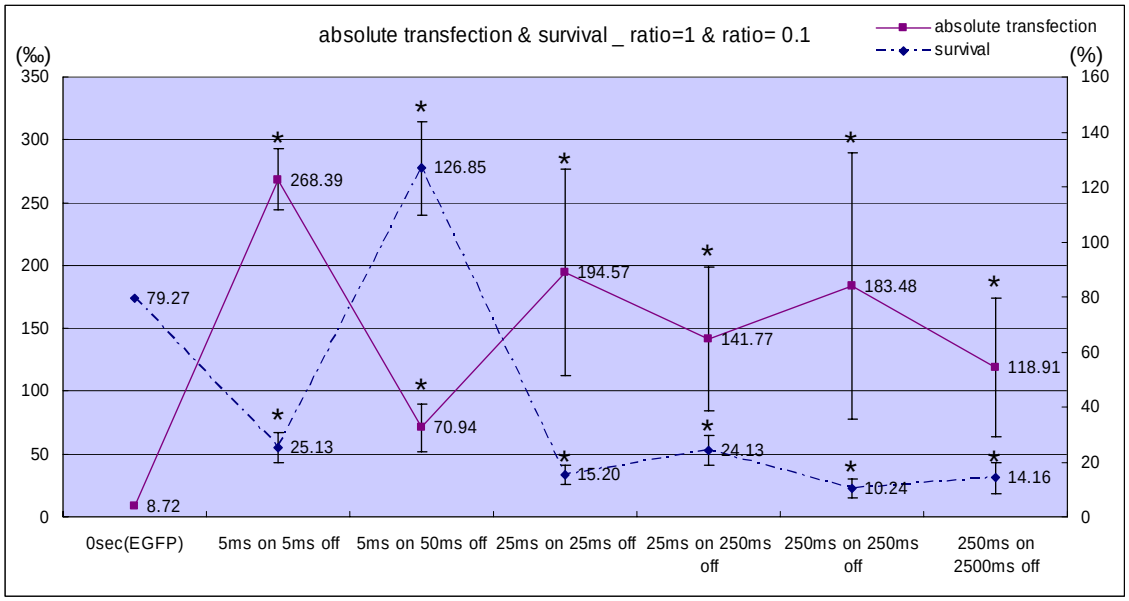

Fig. 2: Dependence of different ratios of the on-time to the off-time on the absolute transfection efficiency and the cell survival (on-time/off-time=1 or on-time/off-time=0.1). Asterisks denote statistical significance of comparison to control (* P<0.05, Two-tail t-test).

Our major results are summarized as the following:

(1) HeLa cells should be exposed for more than 15 sec for effective transfection.

(2) The PRF of ultrasound exposure should not be higher than 4000 Hz for a better gene transfection. It means that the on-time period should not be shorter than 125 μs.

(3) While the PRF of ultrasound exposure is lower than 2 Hz, it has high transfection efficiency but it also induced an extreme temperature elevation.

(4) The optimal PRF ranges from 20 Hz to 1000 Hz (while the PRF is higher than 1000 Hz, transfection efficiency would drop rapidly).

(5) While PRF is between 20 Hz to 1000 Hz, the ratio of the on-time to the off time would better be larger than 0.1, i.e. $\frac{on-time}{off-time} > 0.1$ for a better transfection.

(6) The second ultrasound exposure of the long on-off strategy did not have contribution on the transfection efficiency probably because of the small on-off ratio (on-off ratio <<0.1).

V. 討論

In our study, we found that at a constant delivered acoustic energy, the transfection efficiency varied significantly for different combination of on and off time period and the pulse repetition frequency. For a 50% duty cycle, PRF from 2 Hz to 2000 Hz seems to be a better choice, and the on-time to the off-time should be kept more than 0.1. It would be desirable to know the detailed interaction mechanisms of ultrasound mediated gene transfection between the ultrasound waves and the cells in vitro. Both thermal and non-thermal mechanical effects from high positive and negative ultrasound pressures may contribute to the enhanced gene transfection in vitro. According to previous studies, cavitation may also be the dominant mechanism in our current study. We have tested the effect of medium temperature alone (simulate the ultrasound thermal effect on the transfection efficiency. However, no difference in transfection was observed for temperature ranging between 37 and 43(C. For the future plan, we will focus on if different amount of cavitation was induced in the different combination of PRF and time ratio, and determined the transfection efficiency.

The existence and distribution of cavitation nuclei in tissue are quite different from that in in vitro cell studies. How to increase ultrasound-mediated gene transfection efficiency in vivo, where large free air bubbles do not exist, is still a significant challenge in the field. Several possible strategies can be explored in future studies. First of all, contrast agents that are specially designed for therapeutic use (e.g., larger size and more resilient to break) may be beneficial for ultrasound-mediated gene transfection in vivo. A recent study using two different ultrasound contrast agents with similar albumin shells and perfluorocarbon gas contents demonstrates that perfluorocarbon-exposed-sonicated-dextrose-albumin (PESDA) microbubbles has superior gene transfection efficiency than Optison®(Pislaru et al. 2003). A possible explanation of this observation is that PESDA contains more microbubbles with diameters above 8 μm, and PESDA has less soluble gas (C^4F^{10}) than Optison (C^3F^8). A larger microbubble produces higher shear stress before fragmentation, and may break into larger daughter bubbles that again produce higher shear stresses. Higher transfection efficiency is thus reasonable.

Second, free bubbles can be generated from nuclei by co-treatment of the gas-saturated fluid with a low-frequency acoustic field or a transient high-pressure field (Melodelima et al. 2004) during ultrasound-mediated gene transfection. Modalities such as low-frequency ultrasound, high-intensity focused ultrasound, or shockwaves can be used to ‘cavitate’ bubbles in the

target tissue. Once the treatment ultrasound wave is switched on, the generated bubbles will establish new equilibrium radii and exert sufficient shear stresses necessary for gene transfection. The primary drawback of such a combined treatment strategy, on the other hand, is that the substantially augmented inertial cavitation activity may increase the collateral damage to target tissue and/or transgene, which, however, may be minimized through optimization of the treatment parameters and protocol. The induction of free bubbles for gene transfection may have important implication for in vivo applications since ultrasound contrast agent may not be readily available for the target cells in tissue without breaking the vessel barriers.

VI. 參考資料

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