

Sonochemical and Biological Outcomes of Changing Acoustic Modulation and Their Implication in Therapeutic Ultrasound.

Mariame A. Hassan^{1,2†}, Mikhail A. Buldakov³, Ryohei Ogawa¹, Qing-Li Zhao¹, Yukihiko Furusawa¹ and Takashi Kondo¹ (¹ Graduate School of Medicine and Pharmaceutical Sciences, Univ. Toyama, Japan, ² Facult. Pharm., Cairo Univ., Egypt, ³ Cancer Research Institute of Tomsk Scientific Center, Russia)

1. Introduction

Searching for methods for optimizing “gene delivery” is now the most challenging branch of research after a long list of DNA-based therapeutics have been introduced for clinical development as candidates for treating several malignant as well as non-malignant diseases. The use of ultrasound (US) in mediating macromolecular uptake has proven to be promising, yet; the sonication process embraces multiple variables that make general “optimization” difficult. In such case, a better understanding of the role of each variable over a wide range might be helpful in achieving maximum clinical benefit. Thus, we have undertaken this study to determine the sonochemical and biological impact of changing pulse repetition frequency (PRF) in the range from 0.5 to 100 Hz¹. The underlying acoustic effectors and their effects on macromolecular delivery have been also evaluated².

2. Materials and Methods

2.1. Acoustic setup

1.0 MHz ultrasonic generator (KUS-2S, ITO ULTRASONIC Co., Ltd., Tokyo, Japan) with fixed duty factor of 50% was used. The US generator is capable of operating in the continuous wave (CW) mode and in the pulsed wave mode, and also at wide range of PRFs from 0.5 up to 100 Hz. A planar circular transducer (diameter = 2.7 cm) was fixed at the bottom of a water tank filled with partially degassed water just to cover the transducer surface. Culture dishes (3.5 cm diameter and 1.0 cm height, code # 430165, Corning Inc., Corning, NY) were placed in the near field directly above the transducer and perfectly centered. The attenuation due to culture dish was less than 10%. Two intensities of 1.0 W/cm² ($I_{SATA} = 0.3$ W/cm²) and 1.5 W/cm² ($I_{SATA} = 0.8$ W/cm²) were employed. The time of sonication was doubled for pulsed wave mode at DF 50% to keep the net ultrasound energy for exposure constant. All experiments were carried out at room temperature.

2.2. Evaluation of the sonochemical effects at different PRFs

2.2.1. Detection of inertial cavitation

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) and 2,2,6,6-tetramethyl-4-piperidone (TMPD; dissolved in 50 mM Na₂HPO₄, pH 9.0) were used to trap the hydroxyl radicals (OH[•]) resulting from sonolysis of water to form DMPO-OH adduct and 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN), respectively, detected by electron paramagnetic resonance (EPR) spectrometry (RFR-30, Radical Research Inc., Tokyo, Japan). The relative amount of radicals detected was used as an indicator for the degree of inertial cavitation.³⁾

2.2.2. Assessment of non-cavitation effects

Non-cavitation effects of US were assessed through the extent of sucrose hydrolysis in aqueous solution. Samples of 1 ml were withdrawn at 2, 5 and 10 min and assayed spectrophotometrically by measuring the change in absorbance at wavelengths of 220 and 266 nm using a spectrophotometer (MultiSpec 1500; Shimadzu, Kyoto, Japan). The average peak height at $\lambda = 220$ nm was used for data analysis.⁴⁾

2.3. Evaluation of the biological effects at different PRFs

2.3.1. Assessment of cell killing

Human myelomonocytic lymphoma (U937, suspension) cells and Human cervical adenocarcinoma (HeLa, monolayer) cells have been used in the study. The cells were sonicated at 1 MHz frequency and 0.3 W/cm² intensity for 30 s and 15 s with continuous wave US and pulsed wave US (50% duty cycle), respectively.

Analyses included the evaluation of short- and long-term viability. Trypan blue dye exclusion test was performed immediately after sonication. US-induced apoptosis was evaluated 6 hr post sonication using an apoptosis detection kit (FITC-Annexin V/Propidium iodide double staining) and samples were analysed using a flow cytometer (Beckman Coulter, Miami, FL).

2.3.2. Macromolecular delivery profile

HeLa cells were used to depict the profile of macromolecular delivery in the range of PRFs under study using appropriate reporter agents, namely; 4KD FITC-dextran and plasmid DNA

(pDNA, pGL3-control encoding for luciferase gene) under air-saturated and degassed conditions. The reporter molecules were added immediately before sonication. The cells were then allowed to incubate for either 30 min or 24 hr before the uptake of FITC-dextran was analysed using flow cytometry or the extent of gene expression was determined using luminometry (Turner designed luminometer TD-20/20; Promega Corp.).

2.3.3. Macromolecular delivery optimization approaches

The optimization approaches included the addition the echo contrast agent; Levovist[®]. Levovist[®] was added at a concentration of 1 mg/ml immediately before sonication and washed following exposure.

3. Results and discussion

Our results showed that the PRFs range under study (from 0.5 to 100 Hz) was able to change the characteristics of the US field depending on the on- and off-time length. Using a fixed duty cycle (DC) of 50%, we have observed that at frequencies lower than 5 Hz, the off-time intervals (>100 ms) between pulses were long enough for the relative subsidence of the pulses causing them to look like repetitive mechanical bursts inducing stirring of the solution and limiting the chances for constructive interference with the reflected US beam at the boundary layer due to surface agitation. This was gradually diminishing with increasing the pulse frequency until the solution seemed to be rather stagnant and continuously irradiated at frequencies above 5 Hz. Also, we have found that both chemical and biological effects of US depended greatly on PRF at low intensities. For instance, **Fig.1** shows that the highest OH radical formation was observed at CW while in the pulsed mode, it correlated indirectly to pulse modulation being the highest at 0.5 Hz and decreasing gradually with increasing PRF till a minimum value at 5 Hz. The region above 5 Hz resulted in another phase of significant gradual increase compared to the control. Similarly, cell killing and macromolecular uptake followed that same pattern. Interestingly, the acoustic streaming, despite being equal in magnitude at all parameters tested, the change of its pattern from pulsatile to almost static with increasing PRF played a role in the different uptake levels whereas no effect was observed on sucrose hydrolysis. The macromolecular uptake was shown to be due to enhanced sonoporation and the use of Levovist[®] resulted in differential enhancement of uptake without further increase in cell killing.

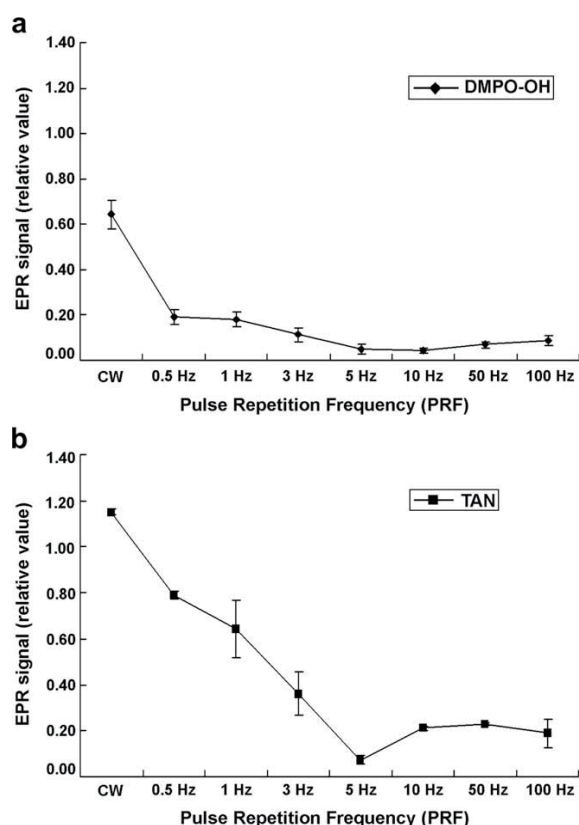


Fig. 1 Detection of the presence of inertial cavitation as a function of pulse repetition frequency (PRF). Hydroxyl radicals resulting from water sonolysis were trapped by (a) DMPO and (b) TMPD. The measurements were done immediately after 4 min sonication at 0.8 W/cm².

3. References

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