Effects of high frequency ultrasound caused by lithium niobate on the intracellular generation of reactive oxygen species

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1. Introduction

In recent years, cancer therapy methods using reactive oxygen species (ROS) have been attracting attention because of cancer-specific cell death induction by ROS[1)]. Cancer treatment by inducing cell death using ROS generation in cells by light[2)] or drugs[3)] has been reported as a conventional method. These methods face the problem of difficulty in stimulating cancer cells effectively. The light-based method is highly invasive, requiring the insertion of optical fibers into the body due to the penetration limits of the laser[3)]. In addition, drug-based methods are less effective due to the small number of intracellular catalysts[3)]. On the other hand, ultrasound avoids these problems. Ultrasound is minimally invasive due to its long wavelength allowing external irradiation. Therefore, ROS generation by cavitation using ultrasound is effective in cancer therapy[4)]. However, ultrasound methods have so far been reported only at low frequencies with wavelengths large enough for cells. In this study, we generated ROS in cells using high-frequency ultrasound with a wavelength the size of a cell (~ 7 MHz).

The aim here is to assess the intracellular behavior of ROS generated by high-frequency ultrasound using fluorescent reagents. In this report, the conditions for ROS generation were first examined. Based on the results of this experiment, the ROS behavior was visualized on the intracellular level.

2. Experimental methods

2.1 Preparation of experimental device

The lithium niobate transducer was made by double side deposition of aluminum lithium niobate plate (**Fig. 1a**). This transducer vibrates when a steel plate and probe electrode turn on electricity (**Fig. 1b**).

2.2 Acoustic Pressure measurements

Acoustic pressure was measured to determine the ultrasound irradiation conditions for the ROS generation evaluation experiments. The original system was built to measure acoustic pressure (**Fig. 2**). This system accurately measures acoustic pressure by removing the noise from the acoustic pressure waveform data acquired with a fiber optic acoustic pressure probe.

2.3 Evaluation of ROS generation

The amount of ROS generated by ultrasound irradiation was measured by 2-hydroxyterephthalic acid (HTA). HTA was a substance formed by the reaction of disodium terephthalate (NaTA) with OH radicals. The fluorescence intensity of HTA (ex: 310 nm, em: 425 nm) was measured by the spectrofluorometer to quantitatively evaluate the amount of ROS generated. The following experiment investigated ROS generation in response to changes in acoustic pressure. First, 7 mL of 4 mM NaTA was poured into a dish. The dishes were then irradiated with the 6.8 MHz ultrasound for 5 minutes under each ultrasound condition. The liquid was collected from these dishes to measure fluorescence intensity by the fluorescence spectrometer.

2.4 Visualization of intracellular ROS

Hydroxyphenyl Fluorescein (HPF) was used to observe OH radicals in cells. The procedures for the visualization of intracellular ROS generation are as follows. First, 4.0×10^4 HeLa cells were incubated at 37 ℃ for 24 hours to attach to a 35 mm glass bottom dish. After washing the dish 3 times with PBS (+), the solution in the dish was replaced with 50 μM HPF and incubated at 37 ℃ for 20 minutes. Lastly, after washing 3 times with PBS (+), oxidative stress was added. The changes due to oxidative stress were observed under the fluorescence microscope for 15 minutes.

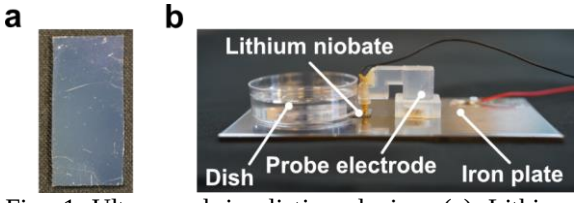

Fig. 1 Ultrasound irradiation device. (a) Lithium niobate. (b) Irradiation device.

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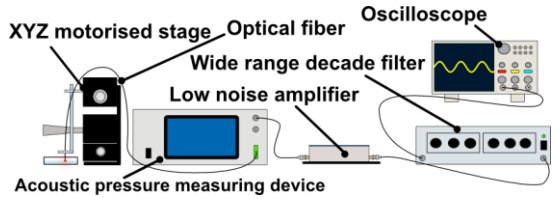

Fig. 2 Acoustic pressure measurement system.

3. Results and Discussions

3.1 Acoustic pressure against input current

The acoustic pressure in the dish was measured every 100 mA. The acoustic pressure increased linearly with the input current (**Fig. 3**). The approximate straight line obtained by the least-squares method is expressed by the following equation (P: Acoustic pressure, I: Input current).

$$P = 0.0024I - 0.5755 \quad (1)$$

3.2 Effect of acoustic pressure on ROS

The fluorescence intensity ratio increased with acoustic pressure (**Fig. 4**). The fluorescence intensity ratio is the fluorescence intensity of each condition divided by the fluorescence intensity of the control condition. The fluorescence intensity ratio corresponds to the amount of ROS generated. This suggested that acoustic pressure influenced the occurrence of cavitation. From these, the ultrasound irradiation of the cells was carried out effectively at an acoustic pressure of 1 MPa for generating ROS.

3.3 Visualization of the intracellular generation of ROS

Under all oxidative stress conditions, the fluorescence observed area changed over time (**Fig. 5**). The control condition was a sample observed for 15 minutes without loading the cells with oxidative stress. The H_2O_2 condition was a sample of the cells loaded with 30 mM H_2O_2. The observation of the sample was carried out for 15 minutes after the loading with H_2O_2. The ultrasound condition was a sample observed for 15 minutes with 1 MPa ultrasound irradiation by the 6.8 MHz transducer. Cell rounding after oxidative stress was suggested to induce ferroptosis by ROS.

4. Conclusion

In this paper, experiments were conducted to study the behavior of ROS on the intracellular. ROS generation experiments showed that the amount of ROS generated increased when acoustic pressure was increased. Furthermore, cellular experiments showed that high-frequency ultrasound irradiation generates ROS in cells. In the future, we will examine the conditions under which high-frequency ultrasound increases the amount of ROS generated on the intracellular.

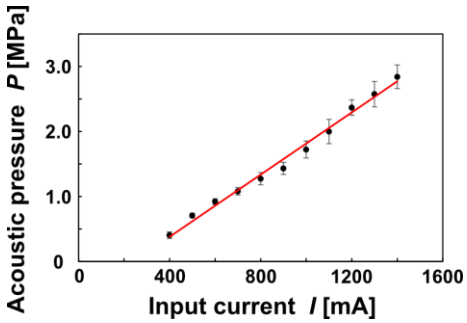

Fig. 3 Acoustic pressure against input current. (n = 10, mean ± S.D.).

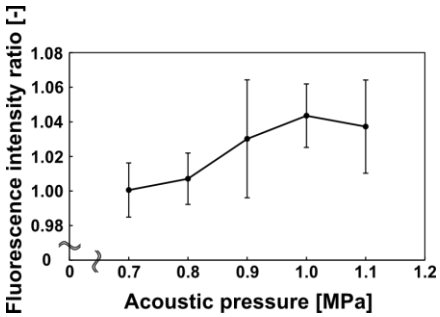

Fig. 4 Fluorescence intensity ratio against acoustic pressure. (n = 3, mean ± S.D.).

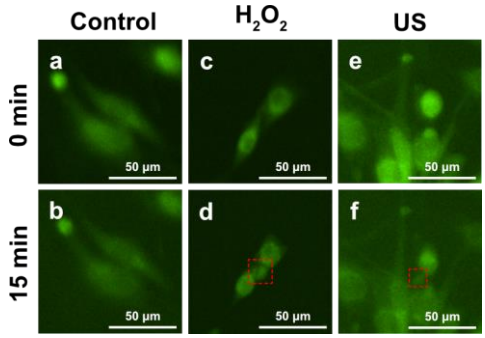

Fig. 5 The generation of OH radical in HeLa cells after different treatments observed by HPF. Red boxes show areas where fluorescence has changed. (a,b) Control sample at (a) 0 and (b) 15 min. (c,d) Oxidative stress sample by H_2O_2 at (c) 0 and (d) 15 min. (e,f) Oxidative stress sample by ultrasound irradiation at (e) 0 and (f) 15 min.

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References

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