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Thaidra A. Gaufin
t_gaufin@hotmail.com

Byron K. Murray

See next page for additional authors

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Authors
Thaidra A. Gaufin, Byron K. Murray, Seiga Ohmine, Kim L. O'Neill, William G. Pitt, and Briant S. Stringham

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Mechanism of Targeted Chemotherapeutic Delivery Using Ultrasound

S. Briant Stringham, Byron K. Murray, Kim L. O'Neill, Seiga Ohmine, Thaidra A. Gaufin and William G. Pitt.

Departments of Microbiology and Chemical Engineering at Brigham Young University, Provo, Utah, USA.

Abstract #1415

Ultrasound (US) is used to enhance and target delivery of drugs and genes to cancer tissues. The advantages of focused delivery to select tissues are manifold, but the exact mechanisms are largely unknown and need clarification to optimize delivery. The present study further defined the role of collapse cavitation in US-induced permeabilization of cell membranes and subsequent drug or gene uptake by the cell. Cavitation, defined as the collapse of micro-bubbles, produces high shear stresses and shock waves that may transiently puncture cell membranes and has been suggested as the cause of increased permeability. The hypothesis that collapsing bubbles permeabilize cells was tested by exposing rat colon cancer cells to US in the presence of excess pressure. Application of pressure suppresses cavitation activity of bubbles without changing the ultrasonic wave; thus if pressure reduces drug uptake, cell permeabilization is strongly linked to bubble cavitation activity. DHD/K12 cancer cells at a concentration of $1x10^6$ /ml in growth media supplemented with 10 μM calcein were placed at the focal point of a 500 kHz transducer. Healthy cell membranes are normally impermeable to calcein, a fluorescent dye used as a model drug in this work. Cells were exposed to 12.8 W/cm^2 pulsed US (1:10 duty cycle) at various pressures and times in an isothermal chamber. The cells were washed to remove any extracellular calcein. Propidium iodide was then added at a concentration of 10 μM. Cell fractions with reversible membrane damage (calcein uptake) and irreversible damage (PI uptake) were analyzed by flow cytometry. The results of these experiments show that increased membrane permeability is proportional to exposure time, and as the cell suspension was pressurized, the calcein uptake decreased as much as ten fold. Pressurization to 3 atm nearly eliminated the effect of US (Fig. 1). Theoretically US could produce thermal and mechanical effects on cells that may enhance drug uptake. These results rule out thermal enhancement of drug uptake and other types of direct US effects. This research shows US-mediated cell membrane permeability is linked to cavitation bubble activity and not thermal effects, thus advancing our knowledge of US-targeted chemotherapy.

Introduction

Ultrasound is currently used for diagnostic and therapeutic purposes. Diagnostic ultrasound utilizes the fact that ultrasound penetrates aqueous tissue and can therefore facilitate imaging of internal body structures. Ultrasound is used to warm targeted tissues in physical therapy applications. The frequencies typically used for diagnostic purposes are greater than 1 MHz; those frequencies for therapeutic use are around 1 MHz. In the current research, a transmitter that functions at 500 kHz was used.

Recently, ultrasound has been used for the delivery of drugs and genes to a variety of tissues. For example, insulin has been delivered transdermally [Mitragotri]. Other drugs and genes have been delivered to specific tissues and locations after intravenous introduction [Pitt]. Ultrasound permeabilizes the membranes of these cells, but the exact mechanism by which this occurs is under investigation. Because the ultrasonic wave is composed of high and low pressure regions, it can act mechanically on tissue. It has been hypothesized that collapse cavitation, as a result of sufficient ultrasonication, is what causes the increase in membrane permeability [Guzman].

At certain thresholds of intensity or resonant frequencies, ultrasound causes oscillation of microbubbles. This oscillation of microbubbles can be defined as stable cavitation. Stable cavitation causes microstreaming, and other mechanical effects, however, it is not considered a major source of membrane permeabilization. When the oscillation becomes vigorous enough, collapse cavitation occurs which releases energy in the forms of heat and powerful shockwaves. These shockwaves are believed to be the source of membrane permeabilization. It is through the use of a pressurized chamber that this study investigates the effects of decreased collapse cavitation on cellular uptake of a model drug. This study investigated the hypothesis that collapse cavitation is involved in permeabilizing cell membranes. Specifically, application of static pressure suppressed collapse cavitation, which in turn will reduced membrane permeability.

Materials and Methods

Reagents: Calcein (622.5 g/mol) (MP Biomedicals, Inc., Aurora, OH) was dissolved into 1X DPBS Ca2+/Mg2+ free to create a stock solution of 0.1 mM stored at 4˚C and sheltered from light. 50 μl of stock solution was then added to 0.5 mL of cell suspension.

Cell Culture: DHD/K12 tRb rat colon cancer cells (ATCC, Bethesda, MD) were cultured at 37˚C, 5% CO_2 in RPMI 1640 media (Gibco BRL, Gaithersburg, MD) and supplemented with 20% cosmic calf serum (Hyclone, Logan, UT). The cells were harvested during exponential and suspended at a concentration of 1x106 cells/ml.

Apparatus: The chamber used for sonicating the cells was an aluminum box of 1 L volume lined with acoustic absorbing rubber and open at the top. Placed on one wall was the 500 kHz ultrasonic transducer. An X-Y stage on top of the box positioned a pressure tube containing the sample at the focal point of the transducer (Fig. 6). Within this tube a small rod held a small polyethylene pipette bulb filled with cell suspension. Pressure was applied to the interior of the tube (and sample) from an air tank at 0, 15, 30, and 45 psi.

Electronics: A sine wave signal at 476 kHz was generated by a HP Waveform Generator, model 33120A. A 100 cycle burst in a 1:10 duty cycle was sent to an ENI Model 240L RF Power Amplifier, which in turn sent the amplified signal through a matching network to the ultrasonic transducer. The signal was monitored on a digital oscilloscope. Power density of the ultrasound at the focal point was measured in preliminary experiments using a calibrated hydrophone.

Flow Cytometry: After sonication, cells were washed three times in 1X DPBS Ca^{2+}/Mg^{2+} free. Propidium iodide (PI) (Sigma Chemical, St. Luis, MO) was added to 0.5 mL of cell solution at a concentration of 10 μM. Cells were analyzed in an Epics XL Flow Cytometer for forward and side scatter, cell fractions with reversible membrane damage (calcein uptake), and those with irreversible damage (PI uptake).

Figure 6. This is a photograph of the sonication chamber. The transducer is on the right while the pressurized chamber and sample holding rod are located in the center.

Results

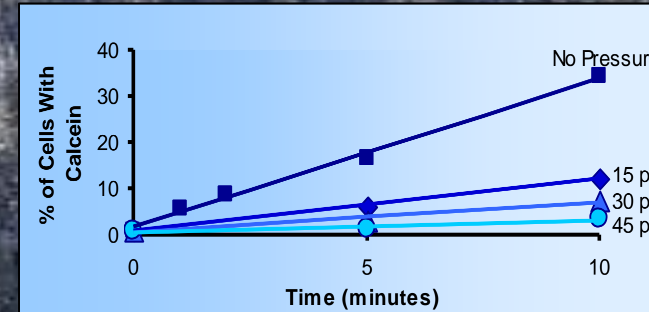


Figure 1. These data represent samples that were sonicated at an intensity of 12.8 W/cm^2. The R^2 values for the linear equations are 0.9961 at no added pressure, 0.9961 at 15 psi, 0.9442 at 30 psi, and 0.7897 at 45 psi.

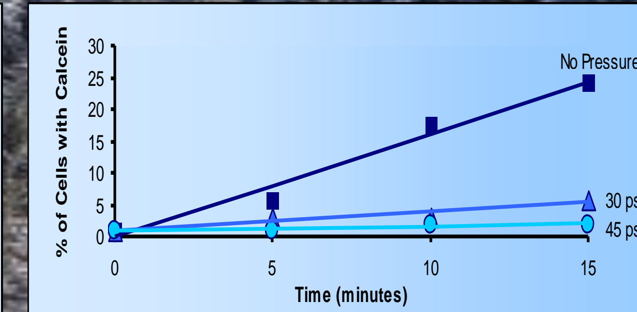


Figure 2. These data represent samples that were sonicated at an intensity of 6.4 W/cm^2. The R^2 values for the linear equations are 0.9739 at no added pressure, 0.8907 at 30 psi, and 0..8124 at 45 psi.

Figure 3. The first two histograms show that pressure alone has little, if any, effect on Calcein uptake when applied in the absence of ultrasound. The third plot shows the increase in Calcein uptake based on added ultrasound. It is the source of the 10 minute data point in figure 2.

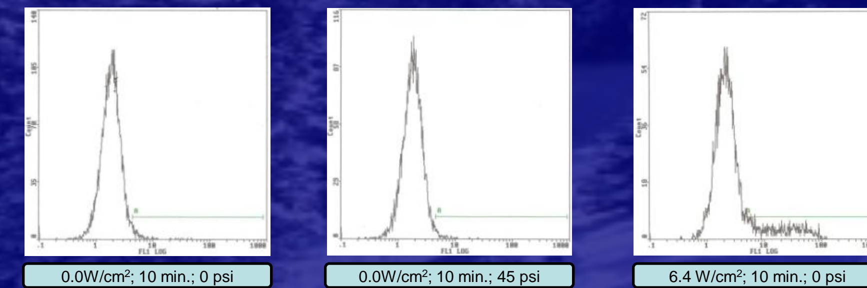


Figure 4. These three histograms show the sharp decrease in Calcein uptake as pressure is increased during 15 minute samples. Notice that at 45 psi, the Calcein uptake is comparable to that in the control samples.

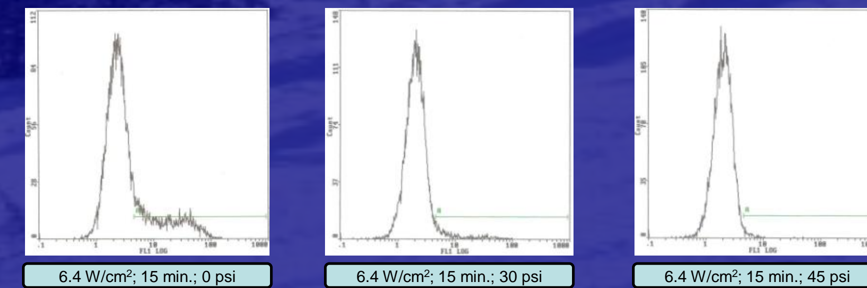
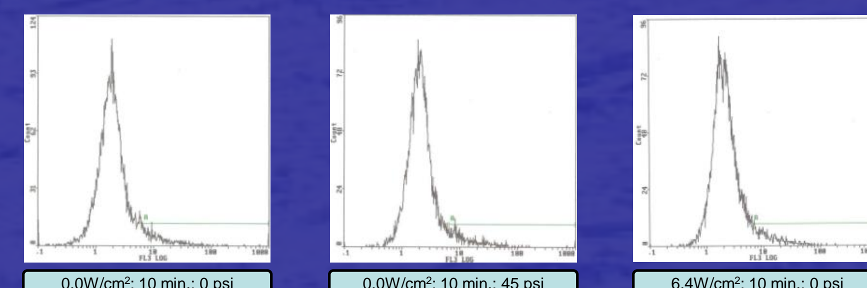


Figure 5. These Histograms represent the level of propidium iodide uptake in control samples versus a sonicated sample. Note that pressure does not appear to increase cell death at 45 psi, and there is not a noticeable increase in cell death when sample was sonicated at noted time and intensity.



Discussion

Collapse cavitation is caused by the oscillatory expansion and contraction of a gas bubble as the ultrasonic wave passes across the bubble. When the oscillations get too large, the bubble contracts so much that the contracting walls slam into each other, producing "collapse cavitation" and its associated shock wave. When the static pressure in the experiment is increased, the bubbles start out more compressed, which in turn decreases the magnitude of oscillation; therefore less collapse cavitation occurs at higher static pressures. Because increasing the pressure was effective in decreasing uptake, the hypothesis that collapse cavitation is the cause of increased membrane permeability is strengthened. The data show that with added pressure of 45 psi and at an intensity of 12.8 W/cm^2, uptake decreased by 89.4% for the ten minutes sample and 93.2% for the five minute sample (Fig. 1). At 6.4 W/cm^2, there was a decrease in uptake of 84.0% at five minutes, 90.3% at ten minutes, and 92.0% at fifteen minutes (Fig. 2). At various pressures, the uptake decreased as the pressure increased.

Furthermore, the data show that calcein uptake is based on time and intensity. Both are important in determining the amount of collapse cavitation and therefore the amount of membrane perturbation.

The data also show that there is a limited amount of cell death at the intensities and frequency used in this experiment as depicted by histograms measuring propidium iodide uptake.

Conclusion

This research has advanced our understanding of the mechanism whereby ultrasound targets drug delivery to cancer cells.

The data indicate that collapse cavitation occurs in the application of ultrasound and is the cause of membrane permeabilization.

The amount of membrane permeabilization is proportional to ultrasonic exposure.

At the experimental times and intensities there was no increase in cell death above control samples.

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