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Synergistic Effects of Ultrasound-Activated Microbubbles and Doxorubicin on Short-Term Survival of Mouse Mammary Tumor Cells

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The primary focus of this paper is to quantify the therapeutic synergism when combining ultrasound, ultrasound contrast agents (UCAs) and doxorubicin for breast cancer treatment using an *in vitro* experimental configuration with mouse mammary tumor (4T1) cells. The 4T1 cells were grown in 96 well plates and allowed to grow to 90% confluency. A 1-MHz focused (f/3) single-element transducer was used to expose the microbubbles (MBs) (Definity) with ultrasound near the surface of the cells. After the ultrasound exposure, different doses of doxorubicin were added and incubated for 24 hours at 37 °C, 100% humidity and 5% CO_2. The efficacies of the drug only and ultrasound-activated MBs combined with drug therapies to kill cells were then quantified by analyzing the cell viability after 24 hours of treatment using the MTT Cell Proliferation Assay. The combined therapy resulted in 60±5.9% of cell viability compared to 82±4.5% when only doxorubicin was used. The cell viability was 72±5.8% when only ultrasound-activated MBs were used with a similar acoustic pressure condition. No significant increase in cell death was observed for higher concentrations of doxorubicin whereas higher peak negative pressure of the ultrasound wave resulted in increased cell death.

KEY WORDS: Chemotherapy; MBs; ultrasound.

INTRODUCTION

Several techniques have been used for neoadjuvant treatment of breast cancer, such as radiation, chemotherapy and hyperthermia. Each of these techniques demonstrated success to different degrees but harmful side effects due to the amount of dose (radiation/chemical/thermal) required for the treatment have also been reported. For example, chemotherapy agents can kill healthy as well as cancerous tissues and tumors can develop chemoresistance.[1] Therefore, combining different therapies has been suggested in order to synergistically utilize the benefits of the different techniques while attempting to minimize adverse side effects.

Researchers have demonstrated that ultrasound can be used as a chemotherapy sensitizer for treating different kinds of cancer.[1-5] Ultrasound-induced collapse of ultrasound contrast agents (UCAs) can be exploited for controlled release of drugs at the targeted lesion and also to permeabilize the surrounding tissue to enhance the effects of chemotherapy.[6] The destruction of microbubbles (MBs) via ultrasound is hypothesized to increase the deposition rate of therapeutic drugs across vessels or endothelial cells.[7] Ultrasound can lower the energy threshold required for cavitation of MBs.[8] The cavitation of MBs through interaction with ultrasound has been demonstrated to enhance sonoporation in cancer cells.[9] Other studies have found that sensitizing the vasculature of a tumor by ultrasound combined with UCAs results in improved efficacy of subsequent radiation therapy.[10] The synergism is hypothesized to be

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caused by the ability of radiation therapies to target the endothelium of tumors resulting in apoptosis of endothelial cells. More recently, Karshafian et al[11] found that the combination of ultrasound-activated MBs and chemotherapy was more effective in inducing cell death compared to chemotherapy alone *in vitro* and deduced that the cell viability after the treatment depends on exposure conditions and cell type. They investigated the long-term cell viability using the clonogenic assay for the combined therapy and found enhanced cell death when combining ultrasound-activated MBs and chemotherapy.

The results from these studies suggest that first exposing cells to ultrasound and MBs can precondition cells to be more sensitive to subsequent therapy. In this study, 4T1 mammary carcinoma cells (a model for human breast cancer) in culture were exposed to ultrasound at different pressures in the presence of Definity (UCAs) followed by various concentrations of a chemotherapy drug, i.e., doxorubicin. The effects of the synergistic therapy were quantified and compared to the appropriate controls 24 hours after addition of the drug. The cell viability for each experimental configuration was assessed using MTT Cell Proliferation Assay.

EXPERIMENTAL METHODS

Cell culture

A mouse mammary tumor cell line (4T1 [CRL-2539]) was purchased from American Type Culture Collection (ATCC, Manassas, VA). 4T1 cells were stored at 70 °C, thawed at 37 °C in a water bath, grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) and antibiotic/antifungal supplements (ATCC, Manassas, VA) and incubated at 37 °C, 100% humidity and 5% CO_2. Cells were grown in 75 cm^2 tissue culture flasks (T-75, Corning Incorporated, Corning, NY). When cells were 80% confluent, they were replated in 96 well plates (BD Falcon, San Jose, CA) for subsequent therapy. Each well in the plate holds 0.37 mL of liquid and has a diameter of 6.4 mm. Approximately 14,560 cells were added to each well such that after incubating the plate overnight, a monolayer of cells with confluency greater than 90% was achieved.

Ultrasound and microbubble exposure

In cell culture experiments, the cell plates were filled with culture medium, covered by a plastic cling wrap to act as an acoustic window and placed inverted in a tank of degassed water. The plastic cling wrap also prevented the solution in the wells from mixing with the water bath. For cells exposed to ultrasound and MBs, Definity MBs were first injected into the cell plates before covering with a plastic cling wrap. Definity contains octafluoropropane and is stabilized by a phospholipid shell. The concentration of Definity in the vial was 120×10^8 mL^{-1} gas bodies. The mean diameter ranges from 1.1 to 3.3 μm, with 98% of the MBs smaller than 10 μm. For cells being exposed to ultrasound and MBs, Definity MBs of concentration 1%/vol were used. For uniform mixing of MBs in the culture media, a mixture of culture media and MBs were first prepared separately and then pipetted into each well.

An ultrasonic transducer was then used to expose the surface of the cell plate. The transducer had a center frequency of 1 MHz, focal length of 2.25 inches and f-number of 3 (Model: E1051, Valpey Fisher Instrument Inc, Hopkinton, MA). The –6-dB beam width at the focus was 4.5 mm as measured using a National Physical Laboratory calibrated polyvinylidene difluoride bilaminar shielded membrane hydrophone (diameter of the active element, 0.5 mm; Marconi 699/1/00001/100; GEC Marconi Ltd, Great Baddow, England). A schematic of the experimental setup is shown in figure 1. The transducer was positioned such that the focal point was at the backside of the plate, i.e., at the cell surface. The trans-

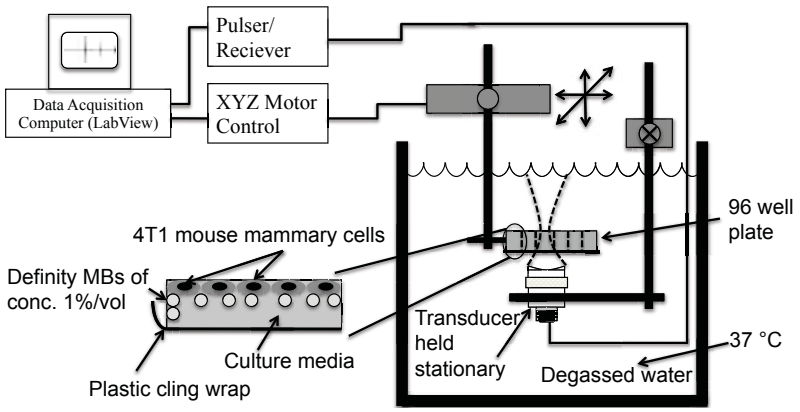

FIG. 1 Experimental set-up

ducer was held stationary and the plate was moved using a computer-controlled micro-positioning system (Daedal, Inc., Harrisburg, PA). The transducer was driven using a pulser/receiver (RAM500, Ritec, Warwick, RI). To obtain smaller changes in the pressure amplitude, a step-variable attenuator was used. The Ritec pulser-receiver generated a five-cycle sinusoidal tone burst with a pulse repetition frequency of 100 Hz. An exposure duration of 30 s per well was used for all the experiments. Rarefactional pressures of 0.5 or 1.3 MPa were used for all the exposures and termed as low and high-pressure settings. Pressure values at the focus were estimated using the calibrated hydrophone and the reflected pressure wave from the wall of the culture plate was used to correct for the estimated pressure value at the cell surface (i.e., the 0.5 or 1.3 MPa).

Chemotherapy

Doxorubicin, a widely used anticancer therapy drug, was used as the chemotherapeutic agent for the study. 4T1 cells were treated with doxorubicin at different concentrations. The stock solution of doxorubicin was prepared by adding 1mg Doxorubicin Hydrochloride (Sigma-Aldrich) to 5.54 mL of deionized water. Here, the following concentrations of doxorubicin were used for the experiments: 5.8 μg/mL, 11.61 μg/mL, 17.42 μg/mL and 34.83 μg/mL. Immediately after ultrasound exposure, the media and MB solution were removed from each well of the plate and the cells were washed with phosphate-buffered saline (PBS). To create uniform concentrations of media and doxorubicin, fresh media and doxorubicin solutions were prepared separately and pipetted into the wells after ultrasound exposure. Generally, there was a time lapse of 6-10 minutes between ultrasound exposure and addition of the drug. The plates were then incubated for 24 hours at 37°C, 100% humidity and 5% CO_2.

MTT cell-proliferation assay

Cell death in the cell culture studies was quantified through the MTT Cell Proliferation Assay. Yellow tetrazolium salt (MTT) is reduced in metabolically active cells, which forms purple-colored crystals that can be quantified by spectroscopic analysis.[12] After incubating the cells for 24 hours postchemotherapy treatment, 20 μL of MTT reagent was added into each well and incubated for an additional four hours. The solution (formazan crystal) in each well was then dissolved with 100 μL of detergent reagent and incubated for another 12 hours. The absorbance was measured using a 540 nm microplate spectrophotometer (ELx800 Absorbance Microplate Reader and Gen5, BioTek, Winooski, Vermont) to estimate the con-

centration of the dissolved purple crystals. The percentage of cell viability was calculated based on the optical density of the solutions compared to the control cell plates.

Trypan-blue staining

In addition to the MTT assay, Trypan-blue staining was also used for qualitative analysis. Specifically, the cell morphologies versus treatment protocol were visualized by adding Trypan blue and then examining the cells under an optical microscope. Trypan blue can also be used to determine cell viability because dead cells take up more stain.

RESULTS

Four different concentrations of doxorubicin and two different acoustic pressure settings were used to investigate the effects of the combined therapy. Based on optical images, the 4T1 cells without ultrasound, MBs and doxorubicin exhibited elongated morphology with intact membranes and no significant amount of cells had intracellular staining as shown in figures 2(a)-(b). The cells exposed to 5.8 μg/mL of doxorubicin had a decrease in the cell proliferation rate attributed to doxorubicin's mechanism of action. Trypan blue staining indicated some abnormal morphology, as shown in figures 2(c), (d). The combined treatment of ultrasound (1.3 MPa), MBs and 5.8 μg/mL of doxorubicin resulted in yet another different visible morphology, where cells did not exhibit elongation, as shown in figures 2(e)-(f). The 4T1 cells were circular with evidence of compromised membranes because some cells exhibited intracellular Trypan-blue staining.

The 4T1 cells from the wells that were treated with ultrasound did not cover much of the well surface area compared to the control and drug only wells. Therefore, it is assumed that the action of the ultrasound and MBs caused many of the cells to become detached from the culture plate surface. When trying to estimate the amount of cell death using Trypan blue, the cells that were detached from the culture plate could not be accounted. Therefore, it was necessary to conduct further assays, i.e., the MTT assay.

The cell viability counts from the MTT assay for the different therapies using different drug concentrations and peak negative acoustic pressures is shown in figure 3. For the concentration of 17.42 μg/mL of doxorubicin and 1.3 MPa peak negative acoustic pressure, the combined therapy resulted in 52 ± 5.9% of viable cells compared to 78 ± 4.5% when only doxorubicin was used. The cell viability was 72 ± 5.8% when only ultrasound-activated MBs were used with similar acoustic pressure. The positive control samples, which did not include any therapeutic agents, were assumed to have 100% viability (for simplicity the results from the positive control samples were not shown in figure 3).

If the observed effects were additive only, then the cell viability should have been approximately 56%. Therefore, there may have been a slight enhancement above merely additive effects in cell death observed when combining ultrasound-activated MBs and doxorubicin to treat 4T1 mammary carcinoma cells. However, it should be noted that the 56% cell death predicted from an additive effect of the 17.42 μg/mL of doxorubicin and 1.3 MPa of peak negative acoustic pressure was within a standard deviation of what was observed at these levels, i.e., 52 ± 5.9%. The mean value of the observed cell death was higher than the predicted but was within the margin of error.

Further examination of the figure does indicate additional trends. First, no significant increase in cell death was observed for different concentrations of doxorubicin. However, higher peak negative pressures of the ultrasound resulted in increased cell death. Therefore, the ultrasound exposure conditions played a significant role in the observed effects.

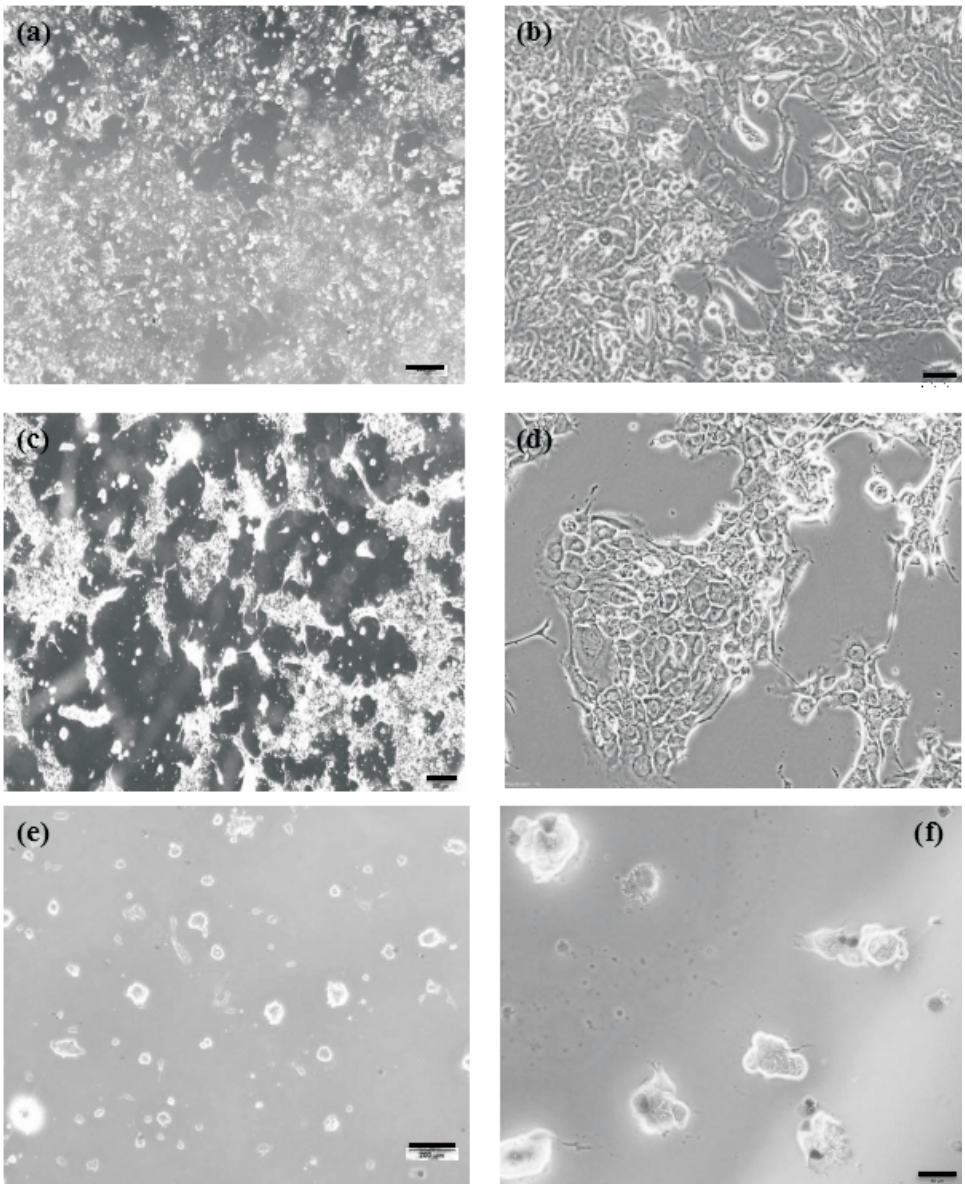

FIG. 2 Light microscopic images of 4T1 cells for (a) control at magnification of 10x, (b) control at magnification of 40x, (c) cells exposed to 5.8 μg/mL of doxorubicin at a magnification of 10x, (d) cells exposed to 5.8 μg/mL of doxorubicin at a magnification 40x, (e) cells exposed to ultrasound-activated MBs followed by 5.8 μg/mL of doxorubicin at a magnification of 10x and (f) cells exposed to ultrasound-activated MBs followed by 5.8 μg/mL of doxorubicin at a magnification of 40x. (The scale bar in the 10x and 40x images are 200 μm and 50 μm, respectively)

CONCLUSION

Synergistic effects of ultrasound-activated MBs combined with chemotherapy were investigated using *in vitro* cell culture experiments. *In vitro* cell culture experiments were conducted by treating 4T1 cells with ultrasound-activated MBs and doxorubicin and compared with the appropriate control and sham exposures. The results from the cell culture studies suggest that the combined therapy additively increased the cell kill. Any enhancement to cell death by combining the therapies would have been small. The results from the 17.42 μg/mL

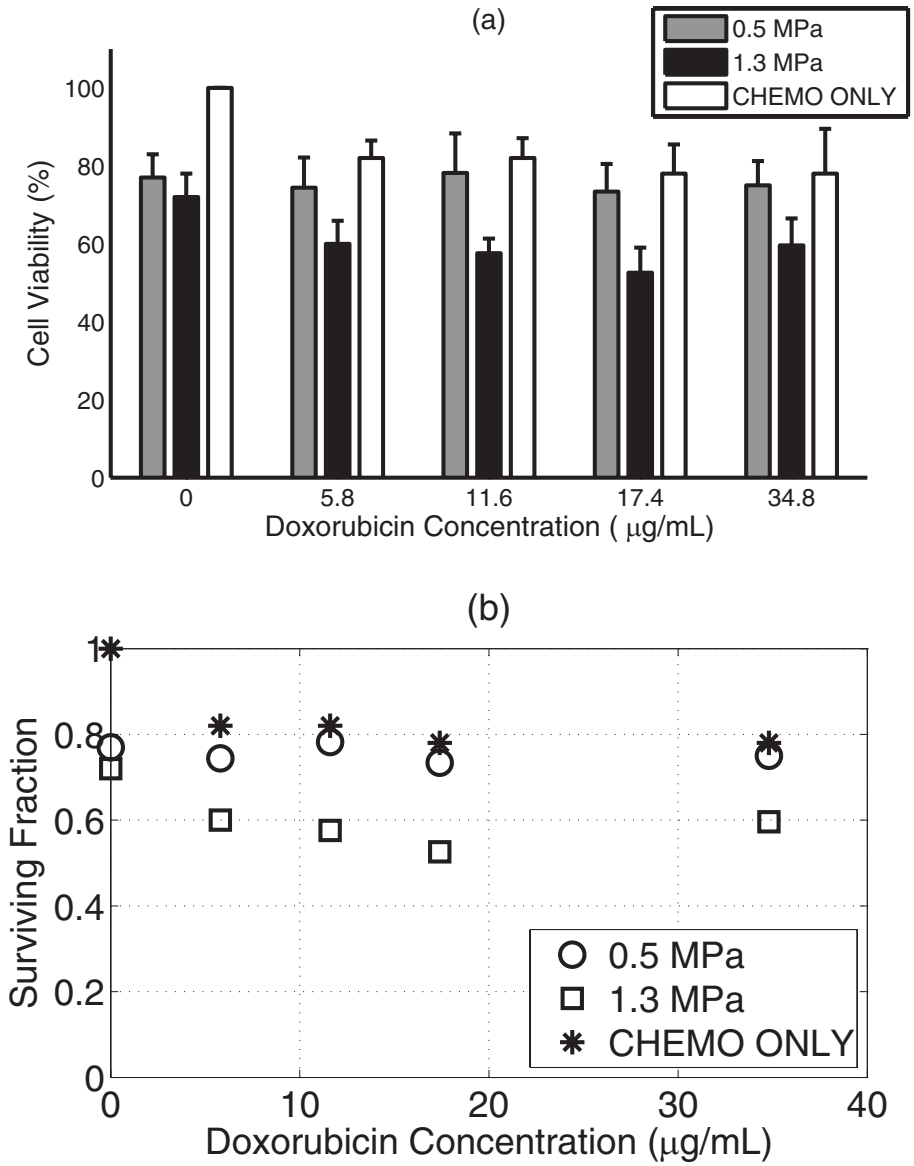

FIG. 3 (a) Percentage of cell viability of 4T1 cells for various experimental configurations. The first two bars of the first four columns represent the results from the combined ultrasound, MBs and chemotherapy and (b) the mean cell surviving fraction for different treatments. MB: MBs, U/S: ultrasound.

of doxorubicin and 1.3 MPa of peak negative acoustic pressure trial suggested a mean effect slightly above additive but within the margin of error for an additive effect. Similarly, for the 0.5 MPa exposures, the expected cell death from the combined therapy would be 23% cell death for ultrasound-activated MBs times another 18% cell death for the chemotherapy treatment or a resulting viability of 63%. Interestingly, from the experimental results the combined therapy at 0.5 MPa exposures resulted in cell viability of 74 ± 7.6%, which does not show an additive effect.

In previous studies using ultrasound-activated MBs to enhance therapy, it was observed that different cell lines reacted differently to the therapy regimen.[11] For example, Karshafian

et al[11] found that the synergistic effects were much less pronounced for the PC3 cell line compared to the MDA-MB-231 cell line. Therefore, our results combined with earlier studies would indicate that the effectiveness of the ultrasound-activated MB combined with chemotherapy will depend on the type of cell line and potentially tumor being treated. In addition, the drug being used may also play a role in the effectiveness of the therapy regimen.

When exposing the cell plates to ultrasound, the entire surface of an individual cell plate was not exposed to ultrasound and MBs. The diameter of an individual cell plate was 6.4 mm and the −6-dB beamwidth was estimated to be 4.5 mm. Therefore, the beam of the ultrasound only covered the center of an individual cell plate and a portion of the cells were not exposed to the ultrasound. For cells not exposed to ultrasound, it is possible that no synergistic or additive effects would be observed. Therefore, the killing effects of exposing cells to ultrasound-activated MBs may potentially be greater than what was assessed in these experiments because some fraction of the cells assayed would only be exposed to doxorubicin. In addition, the cells that were detached from the surface were not assayed as they were removed in the wash after the ultrasound exposure. These cells were also more likely to be damaged or perturbed from the ultrasound and MB exposure. Therefore, the synergistic effects in the cell culture studies are likely higher than what was observed. Hence, future studies will include better targeting of the ultrasound and analyzing only targeted regions with cell viability assays. In addition, ultrasound and MB exposure to nonadherent cell lines will also be examined.

Another factor to consider in future studies is the fluid surrounding the cells during exposure to ultrasound and to chemotherapy. Here we have conducted the experiments by introducing MBs into culture media. We believe that the results reported here might be different if phosphate buffered solution were used instead of culture media because the culture media contains biological substrates that enhance cell growth. Therefore, this could have an effect on chemotherapy response.

One benefit of the proposed method is that the therapy can be spatially targeted to a tumor region. The ultrasound fields used to activate the MBs and perturb the vasculature in tumors can be targeted through focusing to a small volume. Therefore, the effects of the combined therapy can be confined to just the tumor volume and surrounding healthy tissues can be spared. The results suggest that the combined therapy increases the cell death. One of the possible hypotheses for the enhanced cell death by combing doxorubicin and ultrasound-activated MBs may be due to increases in cell membrane permeability and increased shear stress such that effects of the drugs increase. Therefore, if true then the sonoporation effects enhance the therapy. However, because the chemotherapy drug was applied several minutes after ultrasound exposure, it is likely that any transient pores created from ultrasound and MB activity would have resealed. Therefore, other mechanisms besides sonoporation are likely at work in creating enhanced cell kill.

Finally, it was also observed that the highest concentration of doxorubicin did not provide as much cell death as the lowest dose of the drug with ultrasound-activated MBs. In future studies, potential mechanisms responsible for the synergistic and additive effects will be explored along with different chemotherapy drugs and their effects on different cell lines when using ultrasound-activated MBs.

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