Inhibition of Human Breast Cancer Cell Proliferation by Low-Intensity Ultrasound Stimulation

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Objectives—Cancer is characterized by uncontrolled cell proliferation, which makes novel therapies highly desired. In this study, the effects of near-field low-intensity pulsed ultrasound (LIPUS) stimulation on T47D human breast cancer cell and healthy immortalized MCF-12A breast epithelial cell proliferation were investigated in monolayer cultures.

Methods—A customized ultrasound (US) exposure setup was used for the variation of key US parameters: intensity, excitation duration, and duty cycle. Cell proliferation was quantified by 5-bromo-2′-deoxyuridine and alamarBlue assays after LIPUS excitation.

Results—At a 20% duty cycle and 10-minute excitation period, we varied LIPUS intensity from to 100 mW/cm^2 (spatial-average temporal-average) to find a gradual decrease in T47D cell proliferation, the decrease being strongest at 100 mW/cm^2. In contrast, healthy MCF-12A breast cells showed an increase in proliferation when exposed to the same conditions. Above a 60% duty cycle, T47D cell proliferation decreased drastically. Effects of continuous wave US stimulation were further explored by varying the intensity and excitation period.

Conclusions—These experiments concluded that, irrespective of the waveform (pulsed or continuous), LIPUS stimulation could inhibit the proliferation of T47D breast cancer cells, whereas the same behavior was not observed in healthy cells. The study demonstrates the beneficial bioeffects of LIPUS on breast cancer cells and offers the possibility of developing novel US-mediated cancer therapy.

Key Words—breast cancer; low-intensity pulsed ultrasound; proliferation; T47D; therapy

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Abbreviations

BrdU, 5-bromo-2′-deoxyuridine; CW, continuous wave; LIPUS, low-intensity pulsed ultrasound; PRF, pulse repetition frequency; SATA, spatial-average temporal-average; TMB, 3,3′5,5′-tetramethylbenzidine; US, ultrasound

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Cancer is the second leading cause of death in the United States.[1] More than 1.7 million new cases of cancer and 600,000 deaths were predicted in 2019 by the American Cancer Society.[1] Among women, breast cancer is the most common malignancy, accounting for nearly 1 per 3 cancers diagnosed among women in the United States.[1] It is also the second leading cause of cancer death among women.[2] Surgery, chemotherapy, radiotherapy, and hormone therapy are the conventional methods to treat breast and other cancers.[3] Due to the undesired side effects from the conventional therapies, it is crucial to develop new strategies and tools that can supplement conventional treatments.[3] The uncontrolled proliferation characteristic of cancer cells leads to the devastating growth of this

disease.[4] Therefore, inhibiting proliferation can be an effective strategy for novel cancer therapies.

Ultrasound (US), best known for its application in medical diagnostic imaging, can also deliver high-frequency mechanical energy to stimulate thermal and nonthermal bioeffects in cells and tissues and act as a therapeutic tool.[5–7] Ultrasound stimulation of varying intensities and waveforms is currently under investigation for therapy of wide-ranging ailments: fracture healing,[8] painless transdermal insulin delivery,[9] wound healing,[10] enhancing chondrogenesis and osteogenesis of mesenchymal stem cells,[11–13] and treatment of glaucoma.[14] Relatively high-intensity (on the order of 100–1000 W/cm^2) focused US has been used in thermal ablation of solid tumors.[15,16] Unlike such high-intensity methods, low-intensity pulsed ultrasound (LIPUS; 1–100 mW/cm^2) stimulation can generate micromechanical strains and triggers several force-sensitive cellular responses and mechanisms. Low-intensity pulsed US, with a frequency of 1.5 MHz, an intensity of 30 mW/cm^2 pulsed at 1 kHz, and a 20% duty cycle, was approved by the Food and Drug Administration for the repair of bone fractures in the United States in 1994.[17]

Ultrasound stimulation as a potential cancer therapy has been recently investigated using several cell lines at a variety of intensities,[18] including mouse T lymphoma (EL-4),[19] human leukocytes,[20] a human leukemia cell line,[21] and a human myelomonocytic cell line.[22–29] Researchers proposed that defects in apoptosis induction lead to the uncontrollable tumor cell growth; therefore, these studies focused on cell killing through lysis and apoptosis (initiated by cellular membrane damage).[23,28,30–33] However, the effect of LIPUS on breast cancer still remains unknown. The breast cancer cell line T47D is commonly used to model the disease in vitro.[34,35] In this study, the inhibitive effects of LIPUS on the proliferation of T47D human breast cancer cells in a monolayer culture were investigated for the first time to our knowledge, including their dependence on the key US parameters: intensity, duty cycle, and excitation period. To investigate the effects of LIPUS stimulation on healthy breast epithelial cells, MCF-12A cells were chosen to undergo the same treatment. Ideally, a potential cancer treatment plan involving such low-intensity US has several advantages, including causing minimum collateral toxicity to surrounding healthy tissue.[36]

Materials and Methods

Institutional Review Board approval and informed consent were not applicable for this study, as it did not involve human participants or animals.

Cell Culture

T47D human breast cancer cells were cultured in RPMI 1640 media supplemented with 10% (vol/vol) fetal bovine serum, 10-mg/mL insulin, 100-U/mL penicillin, and 100-μg/mL streptomycin. An immortalized breast cell line, MCF-12A, was purchased from the American Type Culture Collection (Manassas, VA) and cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with 20-ng/mL human epidermal growth factor, 100-ng/mL cholera toxin, 0.01-mg/mL porcine insulin, 500-ng/mL hydrocortisone, and 5% (vol/vol) fetal bovine serum. Cells were cultured in a humidified incubator at 37°C with 95% air and 5% carbon dioxide and replenished with fresh media every 72 hours.

Ultrasound Stimulation

Unlike previous studies by other groups,[37–39] to study the effects of varying the different parameters of LIPUS, a custom-designed US exposure system was used (Figure 1). The same setup has been successfully used in our past investigations.[11–13] Briefly, a programmable function generator (33250A; Agilent Technologies, Palo Alto, CA) produced sinusoidal pulses in burst or continuous modes. They were amplified by a broadband 55-dB laboratory radio-frequency power amplifier (A-150; ENI, Rochester, NY) and then supplied to a single-element unfocused immersion transducer (A306S; GE Panametrics, Waltham, MA). A 0.4-mm needle hydrophone (PZT-Z44-0400; Onda Corporation, Sunnyvale, CA) was used to calibrate the pressure generated by the transducer, and the corresponding intensity was determined. The transducer had an outside diameter of 16 mm and a center frequency of 2.25 MHz.

The US transducers and an XYZ positioning stage (Newport Corporation, Santa Clara, CA) were sterilized with 75% ethanol and kept under ultraviolet light for at least 2 hours before experiments were performed. T47D breast cancer cells were seeded at 60% to 70% confluence (7×10^4 cells per well ≈ 18,400

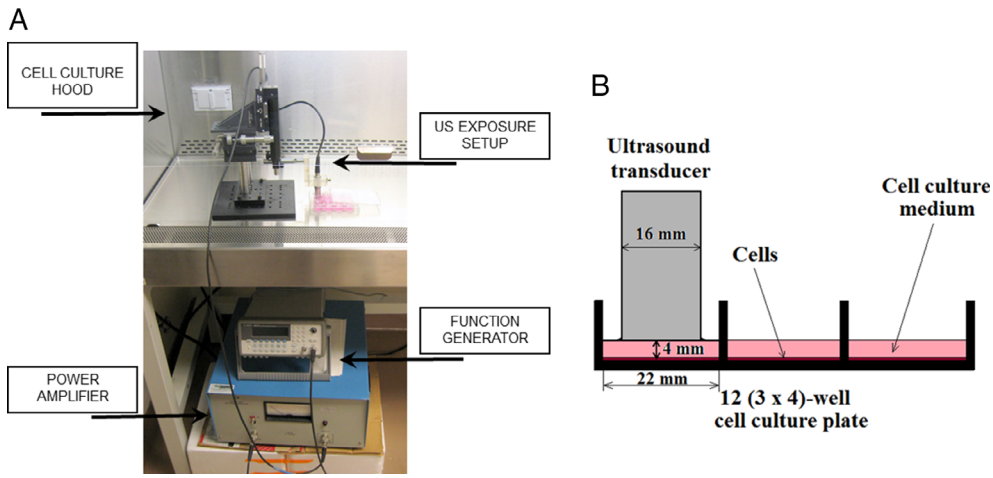
cells/cm^2) on the bottom of a 12-well plate (growth area, 3.80 cm^2) with 1.5 mL of the cell culture medium. The transducer head was positioned vertically over the culture well, just touching the surface of the medium (Figure 1B).

The goal of this study was to investigate the bioeffects of LIPUS on the T47D cancer cells. However, to compare these effects with those of LIPUS on healthy breast cells, we performed a limited number of experiments on MCF-12A cells. These experiments were performed in 24-well plates seeding 3.5×10^4 cells per well to obtain roughly the same cell density as that of the T47D cell experiments. We also use a correspondingly smaller transducer (V323-SU; Olympus NDT, Waltham, MA) with an outside diameter of 6 mm (but the same central frequency of 2.25 MHz) to establish approximate geometric similarity between the two setups.

With knowledge of the cross-sectional area of the cell wells, the volume of the media was chosen so that the distance between the head of the transducer (controlled by the XYZ positioning) and the bottom of the well was approximately 4 ± 0.5 mm. This distance was kept constant for all of the experiments. It should be noted that the cells were in the near field and therefore were subjected to a spatially nonuniform field as in many past investigations of cellular bioeffects of US.[37,38] However, the setup had the advantage of direct stimulation by the immersed transducer unimpeded by an intervening medium, which would otherwise have attenuated the signal. Note that several animal and clinical trials of therapeutic US involved near-field stimulation by transducers in direct contact with the skin.[8,38,40] Li et al[41] specifically found the optimum intensity for far-field stimulations (exposure distance, 240 mm) to be identical to that for a near-field setup (exposure distance, 5 mm). Previously, we investigated the spatial variation of the far and the near fields of the transducer.[42] For the stimulation used here, the spatial-average temporal-average (SATA) intensities were computed by proper spatial and temporal averaging of a spatially varying pressure field for each transducer. The averaging method was described in the Appendix of our previous publication.[43]

For all experiments, cells were seeded on day 0 and received their first US stimulation 24 hours after seeding on day 1. The control group underwent the same experimental treatment with the US powered off. The indirect transfer of US energy in the neighboring wells was investigated by directly measuring the acoustic field in the neighboring well using a needle hydrophone. A transducer positioned for LIPUS stimulation (as described above) was activated in a primary well, and the acoustic field in the bottom of the neighboring well, where the seeded cells would be situated, was measured. It was found to be negligible compared to the energy administered to the primary well. Each cell group was cultured in a different cell culture plate to eliminate the possibility of indirect transfer of US energy from one cell group

Figure 1. A, Customized US exposure system. **B**, schematic representation of the US exposure setup.



to another. There was a possibility of reflections in our setup, which could create a standing-wave pattern, giving rise to a spatially varying acoustic field, as has been noted in recent studies on the effects of US stimulation on drug-bearing vesicles.[44,45] However, note that the cells were restricted here in a monolayer with a dimension that was much smaller than the wavelength. Therefore, the variation of excitation between cells was negligibly small, and the setup was adequate. Furthermore, we investigated the spatial variation of the far and near fields of the transducer as well as the LIPUS intensity without and with passage through a cell culture plate to find that LIPUS reflections at the bottom wall were small in the setup used there.[42] The data were repeated in triplicate for each experimental group.

Determination of Cell Proliferation

The 5-bromo-2′-deoxyuridine (BrdU) enzyme-linked immunosorbent assay (Amersham Cell Proliferation Biotrak enzyme-linked immunosorbent assay system, version 2; GE Healthcare Bio-Sciences Corp, Piscataway, NJ) is based on incorporation of BrdU during DNA synthesis, replacing thymidine, in proliferating cells. To quantify cell proliferation (24 hours after final US stimulation), the BrdU labeling reagent diluted with the cell culture medium (0.4 mL of 1:1000 [vol/vol]) was added to each well of the 12-well plate, and the cells were equilibrated for 2 hours in the incubator at 37°C. The BrdU labeling reagent was then removed from the well, and 0.4 mL of a fixative solution, supplied in the kit, was added to each well. The cells were incubated for an additional 30 minutes at room temperature. The fixative solution was then removed, and 0.4 mL of a 1:10-diluted blocking buffer (also supplied in the kit to block the remaining binding surface and prevent any nonspecific binding of the antibodies) was added to each well. After incubation at room temperature for 30 minutes, the blocking buffer was removed, and 0.4 mL of a 1:100-diluted peroxidase-labeled anti-BrdU (monoclonal antibody from mouse cells conjugated to peroxidase, lyophilized, and stabilized) working solution was added. The peroxidase-labeled anti-BrdU solution was diluted with the supplied antibody dilution solution, and cells were incubated in this solution at room temperature for 90 minutes. The anti-BrdU working solution was then removed, and the cells were washed with 1 mL of a 1:10-diluted wash buffer solution (phosphate-buffered saline, 10× concentrate) 3 times at room temperature; 0.4 mL of a room temperature equilibrated 3,3′5,5′-tetramethylbenzidine (TMB) substrate solution (TMB in 15% [vol/vol]) dimethylsulfoxide) was then added into all wells. The immune complex formed after adding the peroxidase-labeled anti-BrdU reacts with the TMB substrate. After approximately 10 minutes, a light blue solution was obtained, and the reaction was then stopped by adding 100 μL of a 2-M sulfuric acid solution to each well. The optical density (absorbance) of 150 μL of the resultant yellowish solution was read at 450 nm in a 96-well microplate spectrophotometer. The medium containing 0.4 mL of culture medium–diluted BrdU, 0.4 mL of diluted peroxidase-labeled anti-BrdU, 0.4 mL of the room temperature equilibrated TMB substrate solution, and 100 μL of the 2-M sulfuric acid solution was used as an assay blank. The absorbance values correlate directly with the amount of DNA synthesis and thereby to the number of proliferating cells in the culture.

To perform the quantification of cell proliferation on a batch of cells in a cumulative manner (multiple US excitations at an interval of 24 hours) and to corroborate the BrdU assay data, cell proliferation was determined by the alamarBlue assay (AbD Serotec, Kidlington, Oxford, England). This assay is a colorimetric assay. The active ingredient of alamarBlue (resazurin) is a nontoxic, cell-permeable blue compound, which, on entering cells, is reduced to resorufin and produces very bright red fluorescence. The alamarBlue assay was used according to the manufacturer's instructions. In short, a medium containing 10% alamarBlue (50 μL of alamarBlue in 450 μL of the cell culture medium) was added to each well, and the cells were incubated for 2 to 4 hours. The medium containing 10% alamarBlue without the cells was used as a blank. An aliquot of the medium was then withdrawn, and the solution absorbance was measured at 570 nm in a 96-well plate reader. The amount of absorbance is proportional to the number of living cells and corresponds to the cells' metabolic activity. Damaged and nonviable cells have lower innate metabolic activity and thus generate a proportionally lower signal than healthy cells. The calibration curve was constructed

by incubating different numbers of cells with 10% alamarBlue.

Statistical Analysis

In this study, each single experiment was repeated at least 3 times on different passages of T47D breast cancer cells or MCF-12A healthy breast cells. All data are presented as the mean ± standard error. An analysis of variance with GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was performed on the groups to assess the statistical significance. To find individual differences between groups, a Tukey post hoc test was performed between groups. The data were evaluated for normality by the D'Agostino and Pearson test. $P < .05$ was considered statistically significant.

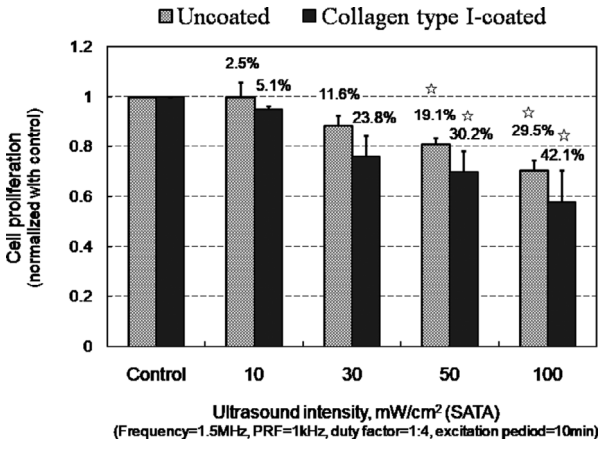
Results

Effects of LIPUS Intensity on T47D Cell Proliferation

The effects of US intensity on proliferation of T47D breast cancer cells were explored by choosing discrete LIPUS SATA intensities: 10, 30, 50, and 100 mW/cm^2. The exposure time was set at 10 minutes, and a frequency of 1.5 MHz, a 1-kHz pulse repetition frequency (PRF), and a pulse duration of 200 microseconds (20% duty cycle) were kept constant. Cell proliferation was quantified by the BrdU assay on day 2, 24 hours after US stimulation. In Figure 2, we show that LIPUS inhibited T47D cell proliferation, and the inhibition was dependent on the intensity of LIPUS stimulation. With increasing US intensity, cell proliferation decreased by 2.5%, 11.6%, 19.1%, and 29.5%, respectively, for the 4 intensities. To rule out the possibility that the inhibition of cell proliferation was because of the detachment of the cells during US treatment, we seeded T47D cells on extracellular matrix protein collagen type I and treated the cells with identical doses of US. Consistent with the result obtained from cells plated on the noncollagen plate, we observed that cell proliferation was inhibited when cells were seeded on collagen type I; it decreased by 5.1%, 23.8%, 30.2%, and 42.1% for the 4 intensities. However, the reason for the increased inhibition with the addition of collagen remains unknown at this time. It indicates that collagen facilitates the bioeffects of LIPUS, but further investigations are needed before any conclusion can be drawn. In all other experiments, for ease, cells were seeded on uncoated 12-well cell culture plates.

The results were further substantiated by an independent cell proliferation assay kit, alamarBlue. The same LIPUS stimulation protocol was repeated, and cell proliferation was quantified 24 hours just before each LIPUS stimulation. Figure 3 shows the results of this time study. On day 1, 24 hours after the cells were seeded and just before the first LIPUS stimulation, the average number of cells in each well was quantified to be approximately 64,000. On day 2, before the second stimulation, the average cell number was again quantified. The control cell number increased from approximately 64,000 to 110,700. The proliferation in treated cells decreased with increasing US intensity by 8.6% (101,200 cells) at 10 mW/cm^2, 20.9% (87,560 cells) at 50 mW/cm^2, and 26.7% (81,190 cells) at 100 mW/cm^2. These values were near those quantified with the BrdU assay. On day 3, before LIPUS stimulation, cell proliferation was again quantified. The number of control cells per well increased to approximately 134,900. With respect to these increased control cells on day 3, 10-minute LIPUS-treated cells showed a minimal

Figure 2. Effect of 10-minute LIPUS stimulation (frequency, 1.5 MHz; PRF, 1 kHz; burst period, 200 microseconds) at different US intensities (SATA) on T47D human breast cancer cell proliferation (normalized with the control) for both uncoated and collagen-coated setups 24 hours after stimulation. The quantification of cell proliferation was performed with the BrdU assay. Stars indicate values significantly different from the control group ($P < .05$).

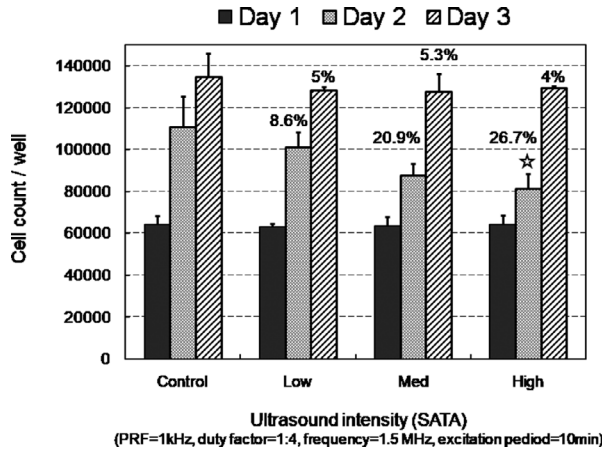


decrease in cell proliferation: 5% (128,200 cells) at 10 mW/cm^2, 5.3% (127,730 cells) at 50 mW/cm^2, and 4% (129,350 cells) at 100 mW/cm^2. However, by then the cells already reached approximately 100% confluence and thus admittedly had a limited scope of inhibition. Note that we were also restricted by a critical starting density: it is well known that cells need to be above such a critical density to be proliferating at their normal rate in absence of any stimulation. The time study was terminated on day 3, and all further experiments were conducted out only until day 2 with a single LIPUS excitation on day 1.

Investigation of LIPUS Excitation on Proliferation of MCF-12A Cells

To compare these effects of LIPUS stimulation with those on healthy cells, MCF-12A cells were subjected to LIPUS with the same parameters and varying intensities. We quantified MCF-12A cell proliferation 24 hours after a single treatment of LIPUS on day 1. The inhibitive effects of LIPUS seen on T47D breast cancer cells were not observed for the MCF-12A cells, as shown in Figure 4. There was no significant difference between the control samples at 10 and 30 mW/cm^2. The proliferation increased when exposed to LIPUS by 20.6% at 50 mW/cm^2 and 29.7% at 100 mW/cm^2 ($P < .05$).

Figure 3. Effect of 10-minute LIPUS stimulation (frequency, 1.5 MHz; PRF, 1 kHz; burst period, 200 microseconds) at 3 different US intensities (SATA; 10, 50, and 100 mW/cm^2) on T47D human breast cancer cell proliferation on days 1 to 3. The T47D cells were seeded on uncoated 12-well cell culture plates, and the quantification was performed by the alamarBlue assay. Star indicates value significantly different from the control group ($P < .05$).



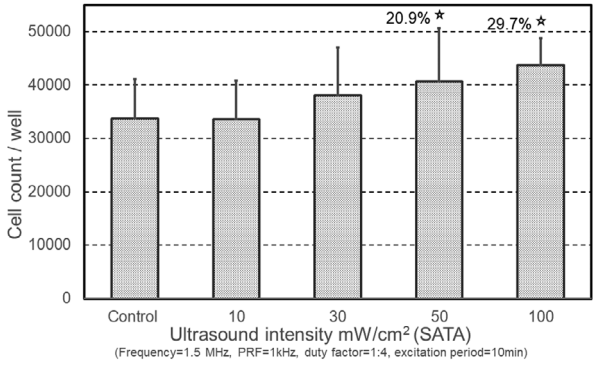
Optimum LIPUS Excitation Period for an Inhibitory Proliferative Response of T47D Cancer Cells

To determine the optimum LIPUS excitation period for a proliferative response, T47D cancer cells seeded onto 12-well cell culture plate were exposed to US at 100 mW/cm^2 (frequency, 1.5 MHz; PRF, 1 kHz; pulse duration, 200 microseconds) for a 5, 10, and 20 minutes. The change in cell proliferation was quantified with the alamarBlue assay. In Figure 5, we show that the US stimulation decreased cell proliferation for each excitation period tested: 14.6%, 36.1%, and 39.4%, respectively, for 5-, 10-, and 20-minute excitation periods. In comparison to 5-minute LIPUS stimulation, the 10-minute LIPUS stimulation significantly enhanced the inhibitory effect on cell proliferation. However, inhibition of proliferation by the 20-minute stimulation was not significantly different from that by the 10-minute stimulation.

Effects of the LIPUS Duty Cycle on T47D Cancer Cell Proliferation

The US duty cycle refers to the percentage of time when US is on during the pulse repetition period. At the spatial-average intensity of 100 mW/cm^2, the effect of the duty cycle on T47D cancer cell proliferation was investigated. The tested duty cycles were 20% (1:4), 40% (2:3), 60% (3:2), 80% (4:1), and 100% (continuous wave [CW]). The quantification

Figure 4. Effect of 10-minute LIPUS stimulation (frequency, 1.5 MHz; PRF, 1 kHz; burst period, 200 microseconds) at different US intensities (SATA) on MCF-12A cell proliferation. Stars indicate values significantly different from the control group ($P < .05$; $P < .01$).



of cell proliferation was performed by the alamarBlue assay on day 2 after 10-minute US stimulation on day 1. In Figure 6, we show that the 20% and 40% duty cycles obtained approximately the same inhibitory effects on T47D cancer cell proliferation. At the 60% duty cycle, cell counts decreased considerably to 35.4% of the control. At the 80% duty cycle, the decrease was greater than 50%, and at the 100% duty cycle, very few cells were counted, indicating complete loss of cell viability.

Figure 5. Change in proliferation of LIPUS-stimulated (intensity, 100 mW/cm^2; frequency, 1.5 MHz; PRF, 1 kHz; burst period, 200 microseconds) T47D human breast cancer cells seeded on uncoated 12-well cell culture plates for different US excitation periods on day 2. The quantification was performed by the alamarBlue assay. Stars indicate values significantly different from the control group ($P < .05$).

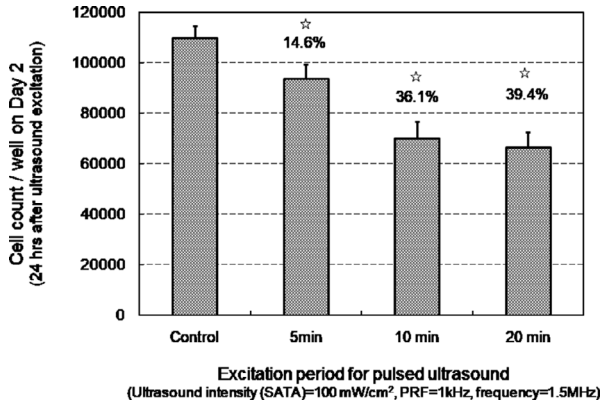
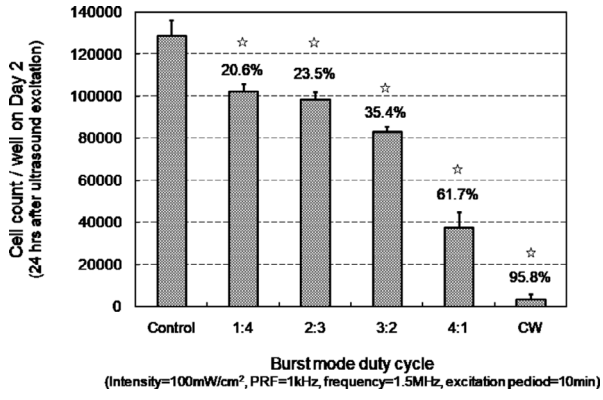


Figure 6. Change in proliferation of LIPUS-stimulated (intensity, 100 mW/cm^2; frequency, 1.5 MHz; PRF, 1 kHz; burst period, 200 microseconds) T47D human breast cancer cells seeded on uncoated 12-well cell culture plates at different duty cycles on day 2. The quantification was performed by the alamarBlue assay. Stars indicate values significantly different from the control group ($P < .05$).



Continuous Wave US Stimulation of T47D Cancer Cell Proliferation

The data in Figure 6 seem to indicate that when the temporal-average intensity of US excitation increases (due to an increasing in the duty cycle right up to a

Figure 7. Effect of CW US stimulation (frequency, 1.5 MHz; exposure time, 10 minutes) at different intensities on T47D human breast cancer cell proliferation. T47D cells were seeded on uncoated 12-well cell culture plates. The quantification was performed by the alamarBlue assay. Stars indicate values significantly different from the control group ($P < .05$).

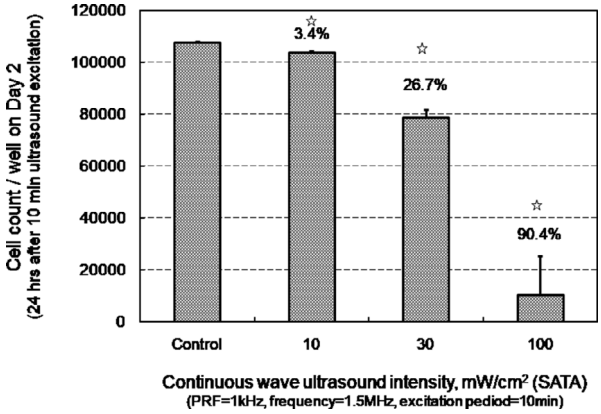
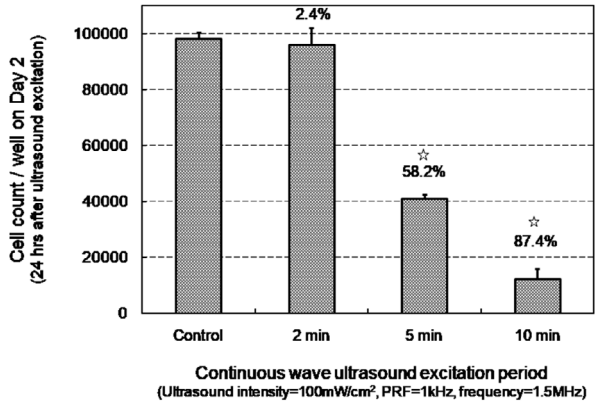


Figure 8. Change in proliferation of CW US-stimulated (intensity, 100 mW/cm^2; frequency, 1.5 MHz) T47D human breast cancer cells seeded on uncoated 12-well cell culture plates for different US excitation periods on day 2. The quantification was performed by the alamarBlue assay. Stars indicate values significantly different from the control group ($P < .05$).



CW form), the inhibitory effect on T47D cancer cell proliferation also increases. However, it would be premature to conclude that US in a CW form is more effective than a pulsed form. To further investigate the CW form, we stimulated T47D cancer cells for 10 minutes by CW US at SATA intensities of 10, 30, and 100 mW/cm^2. Figure 7 shows that, at the lowest intensity of 10 mW/cm^2, the inhibition of T47D cancer cell proliferation was negligible (by 3.4%). At the intermediate intensity of 30 mW/cm^2, the T47D cell proliferation was inhibited moderately (by 26.7%). Figure 6 suggests that the US stimulation in a CW form at lower intensities can generate mechanical stimulation similar to that in a pulsed form at a higher intensity. For example, the effect (26.7% inhibition) of continuous stimulation at a 30-mW/cm^2 SATA intensity was similar to the effect (23.5% inhibition) at a 40% duty cycle of 100mW/cm^2. We further investigated the CW US stimulation at different exposure times. Figure 8 shows that with a decreasing exposure time from 10 to 2 minutes, inhibitory effects of US stimulation on T47D cancer cell proliferation were reduced, from an 87.4% decrease at the 10-minute excitation to a 58.2% decrease at the 5-minute excitation, and finally disappeared for less than 2-minute excitations (2% decrease in cell proliferation).

Discussion

In this study, it was shown that application of LIPUS stimulation can inhibit proliferation of T47D human breast cancer cells in a monolayer culture. In 2 independent assays, BrdU and alamarBlue, decreased proliferation at different LIPUS intensities with a maximum effect at 100 mW/cm^2 was observed (Figure 2). An approximately 30% decrease in cell proliferation could be achieved by only a single 100-mW/cm^2 LIPUS stimulation. The same effect was not observed in the healthy immortalized MCF-12A breast epithelial cells. In fact, LIPUS stimulation of 50 and 100 mW/cm^2 increased the proliferation 20.6% and 29.7%, respectively.

Increasing the duration from 5 to 10 minutes showed an increasing effect, but beyond 10 minutes, increasing the duration did not lead to statistically significant changes (Figure 5). In the literature, this effect has been attributed to a habituation response of cells. Bone cells (osteoblasts) have also been shown to become less sensitive to longer mechanical stimulations.[46] Numerous studies, including one by our group, indicated that a change in LIPUS intensity does not lead to a significant change in its bioeffects at frequencies between 0.5 and 5 MHz.[47,48] Increasing duty cycles of the stimulation from 20% to 40% did not increase the inhibition significantly (Figure 6). However, for 60%, one notices a significant decrease, which culminated (96% decrease in the cell count) for CW US (duty cycle, 100%), indicating complete loss of cell viability. The CW US generated 5 times more mechanical energy (compared to the 20% duty cycle), which could account for the increased damage at the higher intensities. At lower intensities, CW US produced the same effects as the pulsed parameters.

Ultrasound induces a number of different effects: tissue heating, cavitation (formation of tiny gas bubbles in the tissues as the result of US field), acoustic streaming (unidirectional movement in a US pressure field), and acoustic microstreaming (rapidly rotating small-scale fluid motion around oscillating bubbles).[7] In past experiments using LIPUS, the temperature increase induced by the stimulation has been shown to be minimal and can be neglected.[32] Cavitation has been regarded as one of the mechanisms causing nonthermal bioeffects. However, because of the low intensity and thereby low mechanical index ($\approx 10^{-2}$) of the stimulation used, the cavitation threshold is much higher than the parameters tested within this study. Therefore, it can be assumed that the observed effects were nonthermal, and mechanical stimulation caused by US induced inhibition of T47D human breast cancer cell proliferation.

A similar finding of suppression of cancer cell (mouse T lymphoma [EL-4]) proliferation in a monolayer culture due to apoptosis induction after US exposure has been previously reported.[33,49] Note that the result at the LIPUS intensity of 100 mW/cm^2 and 20% duty cycle found in this study were similar to those at 81 mW/cm^2 (SATA), which was previously reported to induce optimal apoptosis with minimal lysis in a human myelomonocytic leukemia cell line (U937).[33] Cancer cells were noted to be more sensitive to US-induced disruption than normal cells,[21,50,51] a finding not in contradiction to our finding that LIPUS, while inhibiting cancer cell proliferation, enhances proliferation of healthy cells.

The observed reduction in cell proliferation could result from cell death due to a number of processes: apoptosis, necrosis, and lysis. In response to various stresses causing irreversible damage, cells naturally go through apoptosis, or cell death, to remove the cells from the environment.[32] Necrosis is when cells die by accidental or inappropriate methods, usually when exposed to harsh environmental conditions.[32] Lysis is a more rapid phenomenon that generally results in breaking down of the cell membrane as a response to shear.[32] Malignant cells, especially breast carcinoma cells, have been shown previously to be more susceptible to US irradiation than healthy foreskin fibroblasts and amniotic fluid epithelial cells.[51] It was hypothesized that the reason that the malignant cells were so susceptible to disruption via US irradiation was because of their fast cell cycle, and US may affect their division.[51] In this study, we showed that LIPUS inhibited cancer cell proliferation and at the same time enhanced proliferation of healthy cells, but the exact reason, eg, cell death by apoptosis, necrosis, or lysis or through disruption of the cell cycle, remains unclear. Further studies are required to elucidate the exact mechanism.

In conclusion, in this study, low-intensity US was shown to induce inhibition of T47D human breast cancer cell proliferation in a monolayer culture. At the same time, it enhanced proliferation of healthy immortalized breast epithelial cells. Our results also suggest an optimum range of key US parameters for the maximum beneficial bioeffects of LIPUS. Cells sense the mechanical force and respond by changing their biochemical activities, resulting in such effects. At this time, the actual mechanism remains unknown and needs to be explored in future research. However, the results are promising and encourage exploration of other nonthermal bioeffects of LIPUS in the future: for example, cancer cell migration. Such investigations are critical for the possible development of US-mediated breast cancer therapy.

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