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Research Article

Examination of high intensity focused ultrasound in Thermal Treatment in Breast Cancer Cells: An Experimental and Statistical Study

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Abstract

Objective : Our study's main goal was to find out how well high intensity focused ultrasound (HIFU) ablation worked in two different cell types of epithelial breast cancer cell lines: 2D monolayers and 3D spheroids. Additionally, the study contrasts experimental data empirically with numerical simulation results utilising a computational model for bioheat. The goal of this comparison is to give a thorough grasp of how acoustic energy is converted within the biological system during HIFU treatment.
Methods : HIFU was used to MDA-MB 231 and MCF7 epithelial breast cancer cell lines that were cultivated in two and three dimensions. During sonication sessions of differing durations, ultrasonication strength and duty cycle (DC) were systematically varied. Bright field and fluorescence imaging of the treated areas were used to measure the temperature elevation and compute the ablation %. The validation of experimental results was conducted using ablation setup simulations.
Results : When HIFU was applied to spheroids with similar duty cycles and acoustic intensities, the temperature increase was less (around 20 °C). DC had a significant impact on the amount of tumour ablation; larger DCs resulted in higher ablation percentages. The length of the sonication, however, had no effect on the level of ablation. These findings were supported by numerical simulations, which showed that the heat was distributed uniformly throughout the grown cells. Spheroids were completely ablated at higher DCs and intensities, but at lower levels, only the topmost layers showed ablation.
Conclusion : According to our research, there is a notable difference in how 2D monolayers and 3D spheroids react to HIFU treatment. Tumour spheroids in particular require smaller temperature elevations for efficient ablation, and increased DC leads to a large increase in their ablation.

Keywords : *HIFU, thermal ablation, 2D monolayer, 3D spheroids, duty cycle, temperature increase.*

INTRODUCTION

After cardiovascular illnesses, cancer is currently the second greatest cause of mortality worldwide, posing a serious threat to public health. Notably, among females, breast cancer is the most well-researched cancer and the primary cause of cancer-related deaths (1, 2). On the other hand, conventional breast cancer therapies include a variety of approaches that depend on the type of cancer, how well the treatment works, and the stage of the disease. These traditional methods of diagnosis and treatment include radiation, immunotherapy, chemotherapy, and invasive surgery, all of which have significant hazards to the health and welfare of patients (3, 4). These treatments frequently involve excision of tumours through surgery or intravenous infusion of chemotherapeutic drugs to target cancer cells that are multiplying quickly (5). Though sometimes successful, these methods' invasiveness results in a number of negative side effects. These can include a poorly defined tumour that damages surrounding tissues (6), problems with medication administration, bleeding, infections, severe illness (7), and a lengthy recovery period following treatment that necessitates prolonged hospital stays (8, 9). An alternate, non-invasive, and non-ionizing method for precisely ablating tumour cells is High-Intensity Focused Ultrasound (HIFU). When applied to a targeted focal region within the body, HIFU can cause coagulative necrosis while sparing other structures that are in the way of the acoustic beams (10, 11). Thermal and non-thermal impacts are the two main biological effects that HIFU generally

generates on cell ablation (2, 12, 13). The process of thermal ablation raises the temperature of the cells to a range of 60–85 °C in a matter of seconds by converting acoustic energy to thermal energy at the target region through an increase in energy density (14). The tumour cells eventually die as a result of the high temperatures in the focused zone, which also cause protein coagulation and cell membrane fusion. Heat diffusion creates a temperature gradient outside of this focused zone, where cells are exposed to temperatures higher than 40 °C without immediately suffering a deadly thermal dose. By means of HIFU waves, acoustic power is transferred to the target region, causing cavitation and ultimately causing mechanical breakage of cell membranes in mechanical ablation, as opposed to non-thermal ablation. High-pressure acoustic waves cause subcellular-scale mechanical injury to tissues by altering the gaseous composition of tissues, inducing oscillation and bubble burst (15, 16).

Patients with malignant tumours of the liver, breast, kidney, and pancreas have responded well to treatment with HIFU (17, 18). Still, there needs to be enough research and analysis done on this technology. Its effectiveness in tumour ablation depends on several aspects, including the target locations and the ultrasonic parameters used, both of which have a big impact on the procedure's success rate. Therefore, in order to maximise treatment outcomes, it is vital that HIFU technology be thoroughly explored and refined. Improving the effectiveness of HIFU tumour therapy requires extensive in vitro evaluations, which include the culture of tumour cells for preclinical cancer studies. Cells are grown in monolayers using conventional cell culture techniques, which reproduce along a single plane. This 2D cell culture model has several benefits, such as affordability, ease of upkeep, and simplicity (13), which makes it a good choice for fundamental cancer research. However, a thorough understanding of the natural behaviour of cells in vivo depends on an accurate replication of the complex interactions between cells and their extracellular environment, which this two-dimensional cell model is unable to provide. These interactions include cellular responses to external stimuli, gene and protein expression, differentiation, and proliferation (19). Researchers use spheroid models to get around the drawbacks of the two-dimensional approach. An extracellular architecture with a layered structure and a proliferative profile can arise because spheroids provide a three-dimensional form that allows cells to interact and proliferate in all directions (20). Oxygen and nutrients are present in this three-dimensional environment, which enables the growth of cells with a gene expression profile that is very similar to tumour cells. As a result, compared to traditional 2D monolayer cell production, the utilisation of spheroid 3D cell shapes provides a more precise assessment of biological responses (21). The purpose of this work is to examine the application of HIFU for in vitro thermal ablation of breast cancer cell lines that are epithelial. These cell lines were grown in three-dimensional spheroidal or two-dimensional monolayer cultures. The investigation examines the interactions between these two cell cultures and sonic waves using both computational and experimental methods. We highlight that 3D spheroids offer a more representative picture of tumour cells undergoing ultrasonic ablation by employing these two different geometrical models. We investigated the effects of ultrasonic parameters, namely duty cycle (DC), sonication duration (SD), and acoustic strength, on temperature elevation and tumour cell ablation extent. Moreover, we employed our computational model for bioheat to compare the outcomes of numerical simulations with our empirical data. The biological system's conversion of acoustic energy is predicted by this model.

MATERIALS AND METHODS

HIFU Mapping and Characterization

Determining the ultrasound transducer's power output per unit volume and focus point was necessary for its characterization. After receiving a sinusoidal electrical signal from a function generator (SIGILENT-SDS 1025), which was pre-amplified by a 50-ohm RF power amplifier (100L Broadband Power Amplifier, Electronics & Innovation Ltd.), the used transducer of a 2 MHz centre frequency (SU101-019; Sonic Concepts) produced HIFU waves. An immersible needle hydrophone (Onda HNR-0500) recorded the mechanical ultrasonic waves that propagated from the electrical impulses, which were generally transformed into them by the transducer. The latter transformed them back into voltage recordings so that they could be processed and quantized into acoustic intensity and then stored in a data acquisition system (22). When the hydrophone was first installed, it was attached to a 3D-motorized axis system that allowed it to move methodically into various locations in relation to the transducer in order to scan a certain geometric volume (Figure 1A). By using this transmission method, the ultrasound transducer's focal point—where the greatest intensity of 5 was recorded—was localised at 51.2 ± 0.1 mm from the transducer's face in the free-field tank that held distilled water that had been degassed (Figure 1B).

Temperature Acquisition

A K-type thermocouple (Omega) was taped over a gel-filled glass slide that was positioned at the focal point within a petri dish in order to quantify the temperature in the focal region of the ultrasound transducer, which was used in the 2D and 3D cell ablation experiments (Figure 2A). Heats this intense could not break the glass slide. The inverted ultrasound probe was covered with a coupling cone that was sealed with an acoustically transparent membrane to deliver ultrasonic waves, with the focal area located just 1 mm from the probe's tip.Using a pump-perfusion kit (PPS2, Multichannel systems), deionized water was constantly pumped in and out of the cone at a maximum flow rate of 30 mL/min in order to cool the transducer and eliminate any possible gas bubbles that might form within the cone. After that, the cone and the petri dish with the glass slide were attached. Additionally, a vertically mounted IR-FLIR E40 Thermal Imaging Camera was placed over the petri dish (Figure 2B) in order to gather quantitative data for the investigation of any temperature variations both inside and outside the focused ultrasonic transducer's focal zone.

2D Tumor Cell Culturing

For this investigation, MDA-MB 231 and MCF7 epithelial breast cancer cell lines were taken into account. The cells were grown in a humidified incubator at 37 °C(23) in a glucose-rich DMEM medium supplemented with 10% foetal bovine serum (FBS) (Sigma, F-9665), 1% penicillin/streptomycin (Lonza, Basel, Switzerland, DE16–602E), and 5% carbon dioxide. This particular medium was abundant in growth factors, nutrients, and antibiotics. FBS served as a source of proteins, and penicillin and streptomycin assisted in preventing bacterial contamination. After 36 hours, the medium was changed again to get rid of any leftovers. Prior to ultrasound ablation, about 3,000 cells were plated on sterile coverslips and cultured for 24 to 36 hours. When the cells reached a confluence of greater than 95%, they were prepared for treatment. From seeding until treatment, the cells were examined under an inverted bright field microscope to make sure there was no contamination and that they were proliferating healthily(24).

3D Tumor Cell Culturing

Using a sphere formation assay, the properties of sphere-like MCF-7 and MDA-MB 231 breast cancer cells were investigated. In this experiment, three-dimensional tumour spheroids that resemble the extracellular matrix (ECM) were created. Single cells were combined 1:1 at a density of 5000 cells/dish with serum-free StableCell TM RPMI-1640 and cold Matrigel, a material decreased in growth factors. Drops of the mixture were added to the microwells in the middle of the small petri dishes, and they were left to solidify for an hour at 37 ˚C in a humidified incubator with 5% CO2. Each petri dish was then filled with 1.5 mL of StableCell TM RPMI-1640 cell growth media with 5% FBS. Until the spheroids were developed enough for additional research, specifically ultrasound sonication, the medium was changed every two to three days. To guarantee uniformity, the cells were incubated for a total of nine days during which time the incubation period was kept constant throughout all tests.

Ultrasound Cellular Ablation

Before beginning any therapy, a bright field microscope was used to capture photographs and check that each petri dish's cells were growing evenly. A glass slide containing nine tiny squares, each measuring 100x100 µm2, was positioned beneath the cells in order to estimate the ablation area. One of these squares was placed in the centre of the focal region, while the remaining eight covered places that were dispersed throughout and outside the treatment zone. Using an ultrasound transducer attached to a cone and positioned in the centre of the glass slides holding the cells, the cultivated cells were subjected to ultrasound sonication (Figure 2C). Following ultrasonic sonication, bright field and fluorescent pictures of cellular ablation were captured at various magnifications (Figure 2D).

Cell Viability Assessment

Trypan Blue Exclusion and Fluorescent Cyto3D Live/Dead Assay were the two staining methods used to assess cellular viability following ultrasonication. A simple and easy-to-use stain called Trypan Blue was used. When applied, it left live cells unstained, while nonviable cells showed a clear blue colour under a microscope. Using Acridine Orange (AO) to indicate damaged cancer cells in red and Propidium Iodide to mark living cancer cells in green, the Fluorescent Cyto3D Live/Dead Assay is a non-toxic nucleic dye (25, 26). Both before and after the tumour cells were subjected to ultrasonication, this dye was administered. The dye needed to be combined with premade media that contained 2 µL of Cyto3D for every 100 µL of medium for both 2D and 3D cell cultures. This concoction was To avoid any signal loss, this mixture was created in a sterile, light-sensitive environment. To enable the chemicals to seep through the gel and into the cell colonies and spheres, 600 µL of the prepared solu-

tion was administered directly to the Matrigel after the media had been removed from the petri dish entirely. This incubation period lasted for at least an hour. An inverted microscope was used to take fluorescent or bright field images for the purpose of visualising cells. To ensure that the ultrasonic therapy was the only cause of the cell death, the viability of the cells was also assessed prior to treatment. On 3D Matrigel cultures, cell quantification was carried out using the subsequent protocol: First, all of the culture medium was taken out completely and put into a conical tube that had previously held media that had been gathered from the petri plate. After that, the petri dish was filled with cold trypsin, which was left to work for around five minutes. The cells were then collected and transferred from the plate to the same conical tube.50 µL of the conical tube's contents were removed and mixed with 50 µL of Trypan Blue after everything had been well mixed. In order to count the cells, 10 µL of the resulting mixture were placed into the hemocytometer.

Statistical Analysis

For each DC ranging from 15% to 55%, ablation data were collected at four spatial-peak pulse-average intensities, ranging from 146.7 W/cm2 to 500 W/cm2. Because the ablation data were not normally distributed and each DC had a tiny sample size, non-parametric tests were used to analyse them using IBM SPSS Statistics (29th edition). For a given SD, many pairwise comparisons using the Kruskal Wallis test and Dune-Bonferroni correction were carried out to identify significant variations in cell ablation between various DCs. Furthermore, the Mann-Whitney U-test was used to compare two separate ablation samples from distinct SDs within a given DC. To evaluate the variations in ablation levels at the focal area produced by SDs of 5 minutes and 10 minutes, the Mann-Whitney U-test was used with all duty cycles. At $p < 0.05$, statistical significance was established.

Computational Modeling

In order to validate the data and forecast the outcomes, we created mathematical models to evaluate the temperature response of both monolayer and spheroid cell cultures to pulsed HIFU acoustic sonication. In order to ascertain the impact of HIFU on cellular ablation, a parametric research was carried out for the various operating parameters, including DC and maximum pressure. The models were verified using experimental data. The distribution of the acoustic field and the accompanying deposition of thermal energy in the cells were obtained from the wave propagation model. The temperature distribution in the cell cultures and the associated ablation rate were calculated using the thermal energy in the thermal model.

Computational Domain

The temperature distribution within the monolayer and spheroidal cell cultures was ascertained using the established model. For this reason, a transducer surface and a petri dish made up the selected computational domain, which is shown in Figure 3A. The petri dish was constructed with many layers for each configuration, beginning with the transducer surface and going through cooling water, cell development media, cells, glass, and the bottom surface of the petri dish. The spheroids cell culture was assumed to occupy a homogenous layer in order to simplify the computational domain. This assumption ignored the spaces that were produced between the various hydrogel spheres in which the cells were seeded. The comparatively modest (~µm) diameters of those spheres make this a viable option. Furthermore, a thin polymeric barrier that separated the cooling water from the cell culture was ignored in the calculations because it is essentially acoustically transparent (27).

Acoustic Field Model

The transducer produced ultrasound waves, which first went through the cooling water and then the culture medium containing the cells (28). A portion of the acoustic energy was absorbed and the remainder was reflected at the interface between each layer. The variation in the acoustic impedance of the different layers (Z (kg/^2s)), which is the product of the layer's density (ρ (kg/m3)) and the acoustic wave velocity (c# (m/s)), determined the fraction of reflected energy (29). Pressure variations within a single layer sheared the cells, increasing mechanical friction, which in turn produced thermal energy (30, 31).

The acoustic pressure field Vp(v, w)(Pa) was calculated in order to represent the propagation of waves inside the cell cultures (32). Since it offered a more comprehensive acoustic field model than the widely used Khokhlov–Zabozotskaya–Kuznetsov (KZK) equation, the Westervelt equation was selected (33). Because it is derived from the equations of fluid motion, the Westervelt equation is more accurate than the KZK equation, which has a validity region (34). A model like this has been widely used in medical ultrasonography since it incorporates the effects of diffraction, non-linearity, and absorption mechanisms seen in biological material (35) and provided by: The loss brought on by

the fluid's viscosity and heat conduction is expressed in the first term of equation (1). The propagation wave's nonlinear distortion is described by the second term, while linear lossless wave propagation is represented by the final two terms. The following is represented by the various terms in the equation: The coefficient of nonlinearity, function of the nonlinearity parameter 8B9 of the medium, and the Laplace operator is represented by 2 (m-2). The absorption/attenuation coefficient (Np/m)) and the angular frequency of the source determine the acoustic diffusivity, which is represented byThe frequency-dependent power law provided by (36) determines the latter where η is the power law's attenuation exponent the attenuation coefficient at a reference frequency of 1 MHz, depending on the kind of medium (37). Equation 3 was used to calculate the heat (thermal energy) deposition from the ultrasonic wave resulting from the acoustic intensity and the medium impedance (Z) after the acoustic pressure was established at each point in the cell culture domain. The model's initial acoustic pressure condition was set to zero at the start of sonication. Additionally, a set of boundary conditions was required in order to solve for the acoustic field. The computational domain was reduced by the design of the petri dish, enabling an axisymmetric solution around the z-axis. Thus, a symmetry boundary condition was selected at planes x=y=0. An explanation of the pulsed HIFU beam produced by the transducer at z=0 (38) can be found in the following formula: where P is the pressure at the transducer surface, and N is the number of burst cycles with a period of TBD (s) each during the entire sonication duration. The aperture angle, γ (rad), is a function of the transducer radius, (m), and the focal distance, d (m). The radial distance from the transducer centre, r (m), is determined by these two factors.

Lastly, because the computational domain was finite, artificial absorption was used to regulate the acoustic reflections from the end of the domain by adopting the perfectly matched layer boundary condition at the petri dish's bottom (end surface) (39).

Thermal Model

The thermal model could then be used to determine the temperature distribution when the acoustic pressure in the petri dish was set. In these applications, the energy balance found in Pennes' (40) bioheat model was frequently employed (32). The model, which took into account both the metabolic heat generation (W/m3) and the impact of blood flow in biological media, was based on the Fourier law. Thus, using equation (5), the temperature distribution was calculated.

where k (W/m√K) and C (J/kg√K) represent the medium's thermal conductivity and specific heat capacity, respectively. The specific heat capacity, perfusion rate, and temperature of the blood are represented by the values J/kg√K, kg/s√m3, and K, respectively. Because there are no blood arteries in the culture-growing medium, blood perfusion has no influence on the cell culture in the petri dish. Furthermore, because the thermal energy from the sonic wave contributes far more to the total energy balance than does the metabolic heat generation, it might be disregarded (32).The starting temperature was chosen at 37 °C in order to solve the bioheat equation. Furthermore, Dirichlet boundary conditions of 25 °C were established for both domain end surfaces, and symmetry boundary conditions were taken into account for planes x=y=0 (41).

Numerical Solution

Utilising the finite volume approach with implicit Backward Euler temporal scheme and central difference scheme for second order spatial differentials, the mathematical models of the temperature distribution and wave propagation were solved. Equation (1) was solved for a transient 3D Cartesian grid using the method described by Doinikov et al. (34) for the nonlinear and absorption terms. The time step was adjusted to Δt=0.01/c# with a grid of Δx=Δy=0.2λ and Δz=0.1λ (42), where λ (=c#/f#) (m) is the wavelength of the transducer's driving frequency in order to guarantee accuracy of the results with the least amount of computational time. The acoustic intensity and the associated thermal energy produced in the cell cultures were measured once a uniform pressure field was achieved at steady state (no change in the acoustic pressure with time). and applied with a time step of Δt=0.01 s in the thermal model. To cut down on computation time, different time steps were used for the thermal model as opposed to the acoustic field model (32). When the estimated residuals of the various parameters between two successive iterations were less than 10-8, the parameters were said to have found convergence. Table 1 displays the various thermal and acoustic characteristics of the monolayer and spheroid cell cultures in addition to the many layers the acoustic wave was travelling through. It should be noted that, as is the case for the majority of biological tissues, the missing sonic parameters for either the cells or their growth media were accepted similarly to those of water (29, 43).

The constructed mathematical model's solution adhered to the flowchart shown in Figure 3B. The thermal and acoustic parameters and arrangement of the cell cultures (monolayer,

spheroid), the transducer characteristics (a, d, c#, f#), and the operating circumstances (TBD, DC, SD) were all inputted into the model. The convergent steady state acoustic pressure distribution from the acoustic field model was obtained by first initialising the temperature and pressure fields. The latter was used to calculate the thermal energy deposition which was then entered into the thermal model to get the temperature distribution that converged.

RESULTS

Focal Area and Temperature Measurements

The focal area changed as the input voltage increased at the horizontal mid-plane. The focal area grew from 3.5 mm2 to 5.2 mm2 when utilising a 5 MHz focused ultrasound transducer, and the input voltage increased from 110 mV to 160 mV (Figure 4A). Using a thread-head K-type thermocouple placed at the ultrasonic transducer's focus area and sonicating at various voltages and duty cycles, temperature readings were taken in real time. The temperature at the focus region rose in tandem with a rise in the ultrasonic waves' duty cycle as the input voltage was changed (Figure 4B). The temperature increased monotonically, reaching a maximum of 81.9 °C when sonicating at an input voltage of 350 mV and a maximum DC of 45%. The minimum temperature was recorded at 43 °C at the lowest input voltage of 300 mV and a DC of 10%. With 10% DC ultrasonic waves and a 350 mV input voltage, the IR camera images highlighted a consistent distribution of thermal energy throughout the focal region of a recorded 134.5 mm2 area.

Cellular Ablation Using HIFU

On 2D Cultures

A 2D layer of cells planted on a glass coverslip, resembling a monolayer of grown cancer cells, was the target of ultrasound sonication. In contrast to an insignificant 3% ablation outside the focal zone, HIFU of frequency 2 MHz at a 35% duty cycle and intensity 280 W/cm2 generated a 95% ablation of the 2D grown cells inside the focal region. The difference in the ablation percentage was highlighted by bright-field images of the cultured cells taken before and after ultrasound sonication for 10 minutes (Figure 5A). The maximum temperature of 60.9°C was reached inside the area of focus, which is about 1.5 times higher than the temperature outside the transducer's focus (Figure 5B). The ablation percentage was influenced by the duty cycle and sonication length of ultrasonic waves in relation to the applied spatial peak pulse average ultrasonic intensity (Figure 5D). A 15% low duty cycle and a 55% high duty cycle produced overlap in the ablation percentage, which changed continuously as *ISSPA* rose, regardless of the length of the sonication. Nevertheless, all cells were destroyed at a 55% DC, whereas fewer than 5% were destroyed at a 15% DC. Contrary to increasing the duty cycle and *ISSPA*, increasing the sonication duration did not always result in a significant change in ablation. The complete ablation of cultivated tumour cells happened at a 35% DC and at the maximum *ISSPA* of 500 W/cm2. (Figure 5C).

Regardless of the length of sonication time used, thermal ablation of malignant cells grown in two dimensions often began at temperatures of around 60 °C and reached full ablation at temperatures above 80 °C (Figure 5E). The temperature and ablation levels attained were not significantly affected by the duration of the ultrasonic treatment (p=0.557). With a rise in temperature, the percentage of ablated 2D cultivated cells grew monotonically.

On 3D Cultures

A 2 MHz ultrasound was used to sonicate cancerous cells that were 3D grown in Matrigel. The ultrasound intensity, duty cycle, and duration were all adjusted. After 10 minutes of sonication with 35% DC ultrasound waves at an intensity of 280 W/cm2, bright field images and fluorescence staining of the cells revealed a significant 90% ablation percentage of tumour cells inside the focal region, raising the temperature to 61.4 °C, compared to a minimal 4% ablation percentage of cells recorded outside the field of focus, achieving a temperature that was approximately 1.57 times lower (Figure 6B). Clusters of dead cells were visible after sonication, reaching up to 99% ablation at a high DC of 55%, according to bright-field and fluorescent pictures magnified 20 times (Figure 6A).

The ablation % as a function of DC and *ISSPA* was influenced by the ultrasonic treatment period. An SD of 10 minutes resulted in higher ablation percentages than an SD of 5 minutes at all parameters used. Regardless of the applied ultrasound intensity, full ablation of malignant cells 3D grown in Matrigel happened at the maximum DC employed of 55% after 10 minutes of ultrasound sonication (Figure 6C). Even with increasing SD and *ISSPA*, a low 15% DC resulted in very little ablation (less than 20%).Although the proportion of ablated tumour cells increased generally with increasing DC, SD, and *ISSPA*, there was less dependence on SD at low and high DCs. This was not the case for DCs in the medium range (35%) where spheroids reached higher ablation percentages as a function of both SD and *ISSPA*. Sixty-five percent of the trials ablated malignant spheroids at

a temperature ≥ 60 °C at the end of each ultrasound sonication (figure 6D). As the sonication time increased, even greater ablation percentages and final temperatures were reached. At temperatures beginning at about 45 °C, minimal ablation of spheroids was seen, with the temperature increasing in a monotonically increasing pattern.

Spheroid vs Monolayer Thermal Culture Ablation

The percentage of ablated cells was dramatically increased by increasing DC in both the spheroidal and monolayer forms. Even at the lowest intensity, a DC of 55% induced the ablation of more than 90% of the cells, whereas a DC of 15% was the least effective. The results showed that for both the spheroids ($p = 0.028$ and 0.022 for SD = 5 and 10 minutes, respectively) and monolayers ($p = 0.008$ for both SD = 5 and 10 minutes), 55% of DC was compared to 15% of DC. But comparable ablation percentages were obtained in both configurations—as long as SD was taken into account—with no discernible variation at any particular DC and The Mann-Whitney U-test produced negligible findings when compared to aring ablation percentages between five and ten minutes' SD, at fifteen percent DC (monolayers: $p = 0.2$; spheroid: $p = 0.4$), and at fifty-five percent DC (monolayers: $p = 1.0$; spheroids: $p = 0.2$).

Tumour cells cultivated in Matrigel took on a 3D spheroid structure; ultrasound sonication of these cells revealed their ablation at temperatures 20 °C higher (Figure 7A). For spheroids to achieve the same ablation percentage at all applied ultrasonic intensities, lower temperatures were required than for monolayers. Furthermore, less scattering was observed with spheroids compared to monolayers of cells in the connection between temperature and percentage of tumour ablation, highlighting the fact that spheroids are less susceptible to temperature variations. Fluorescent Cyto3D Live/Dead Images When sonicating at low DC values, the assay of spheroids after ultrasound sonication revealed partial ablation of cultured cells, which is characterised by the death of the outermost layer (stained in red) while the innermost layer remained intact (stained in green). Nevertheless, total ablation of spheroids was obtained at the conclusion of ultrasonic sonication with higher DC and *ISSPA*, as shown in Figure 7B, where the majority of cells in each cluster—whether in the innermost or outermost layer—became fully ablated after absorbing more power per unit area.

Numerical Simulations

The obtained experimental and numerical findings are shown in this part for the various DC and transducer pressure levels under operation, together with an analysis of their impact on the ablation rate for the various cell culture types (monolayer and spheroids).The created mathematical models were first verified using the two cell cultures' experimental data. In order to achieve this, the model was simulated using the dimensions of the transducer that was used in the experiment, which had a fundamental frequency of 2 MHz, an exterior diameter of 33 mm, and a focal distance of 51.4 mm. In terms of the maximum temperature reached at the focal region within the cell cultures, our computational model describing the ablation of 2D and 3D cultured cells showed agreement with the experimental results with a maximum error of 15%, as shown in Table 2, which is deemed acceptable in practice. Surface plots covering one quarter of the disc in the monolayer and spheroid simulations showed a consistent distribution of thermal energy at low pressure runs of 0.433 MPa, with a negligible temperature gradient (less than 0.1 °C) between the focal region and the remaining culture medium. Both low and high DC levels were used to achieve this (Figure 8A&B). However, a significant temperature gradient was observed between the focal region and its immediate surroundings at high pressure runs of 0.661 MPa. This gradient reached up to 21.7 °C, computed at a high DC of 55% in the monolayer configuration, and up to 14 °C, computed at a DC of 30% in the spheroid configuration (Table 3). However, the temperature distribution was comparatively homogeneous with a small temperature gradient within the focus region.

The way the grown cells interacted with the heat energy produced by ultrasound sonication was unaffected by their arrangement. When it came to their temperature reactions to the sonication settings, spheroid and monolayer cell cultures exhibited comparable behaviours. For the identical sonication conditions, the monolayer culture did, however, reach higher temperature levels than the spheroids. As the experimental investigation showed, there was a notable discrepancy between the temperature achieved by spheroids and monolayers, with differences of up to 20 °C. As DC increased, the temperature differential became more noticeable.

Discussion

The great precision, low invasiveness, and affordability of high intensity focused ultrasound have made it a popular new therapeutic option for cancer (44). In order to investigate the impact of cell configuration and ultrasonic parameters on the effectiveness of HIFU for ablating breast cancer cells, our research concentrated on the effect of pulsed HIFU on 2D and 3D breast

cancer cell culture models. If an HIFU treatment produced an ablation percentage of 90% or higher, it was deemed effective. We investigated the thermal ablation of 2D and 3D cancer cell cultures in detail using a range of duty cycles, sonication durations, and ultrasound intensities. The percentage of cellular ablation and the highest temperature attained after ablation were important indicators. We sought to determine the ideal ultrasound parameters required for successful cancer cell ablation by evaluating how these two culture models responded to ultrasonication. In addition, we created a mathematical model to clarify the response of cells in spheroid and monolayer cultures to pulsed HIFU. The accuracy of the model was further verified by comparing these simulations with experimental data. Our understanding of HIFU's potential for cancer treatment has been significantly enhanced by this integrated method, which allowed us to anticipate cellular ablation results under varied settings, including changes in duty cycles and maximum pressure levels.

Duty Cycle Affects Degree of Cellular Ablation

In ultrasound sonication, the duty cycle is the portion of the burst period (also known as the ON area) where the ultrasonic amplitude is nonzero. An increased DC results in a longer time for cells to absorb energy, which raises the energy input per unit area. On the other hand, a lower DC results in a shorter ON and longer OFF period, when the absorbed energy disperses into the surrounding control volume that surrounds the target. The DC of ultrasonic waves had a significant impact on the percentage of ablated tumour cells in both monolayer and spheroidal configurations, and this effect increased as DC increased. Zhu et al. (45) observed a notable alteration in the lesion's size and structural form while applying a 40 kHz frequency differential between the inner and outer loops of a dual-frequency transducer at 160 W ultrasonic power in vitro on bovine liver tissue. Low DC values, between 5 and 20 percent, did not show any signs of coagulation necrosis; in contrast, coagulation necrosis was seen at DC values of 30 percent and higher, with the maximum ablation percentage at DC of 50 percent. Likewise, our analysis revealed that a low DC of 15% was completely ineffective, whereas more than 90% of the tumour cells were abated at a DC of 55%. As more shock wave arrays continued to strike the targeted tissue, increasing DC lengthened the heating period and decreased the dissipation time, which was characterised by a shorter inactive treatment interval and allowed thermal ablation to take precedence (46). In fact, longer heat depositing intervals led to higher temperatures being reached with larger DC values. There might be a trade-off, though. Previous study has indicated that greater DC values, which produce an increased ultrasonic pulse frequency, increase the likelihood of causing mechanical disintegration and localised fragmentation of the targeted tissue due to inertial cavitation (10). The efficiency of thermal ablation starts to decline after a certain point, even with further increases in DC values beyond those used in our work. At that point, mechanical ablation becomes the more effective method. According to our research, using ultrasonic sonication for five or ten minutes produced almost identical ablation percentages, with very little variation, especially at low and high DC values for both monolayers and spheroids. Based on this result, it was hypothesised that cells could attain a condition of equilibrium in terms of energy dissipation and power absorption prior to the end of the sonication session, indicating behavioural saturation at an ideal exposure duration. As a result, a 5-minute session may last .

Temperature of Spheroid vs. Monolayer Ablation

Spheroid-modeled cells were ablated at a threshold temperature that was about 20 °C lower than that of monolayers, indicating that spheroids are less sensitive to thermal ablation than monolayers. The distribution of nutrients and oxygen in each cluster varied between the two cell shapes. In order to further dissipate energy to the surroundings during the OFF area, the cells during treatment absorbed the sonic intensity in the ON zone. The cell growth medium of spheroids often has a higher thermal conductivity than that of monolayers. Additionally, their surface-to-volume ratio is higher. In contrast to cells in monolayers, where the dissipated power would primarily flow across to the surrounding cell growth media, cells in the innermost core of the spheroids would thereby dissipate power into the surrounding cells during the OFF area.

The findings demonstrated that in both cell arrangements, the focal region's heat was distributed uniformly. On the other hand, spheroids recorded a maximum intensity that was 12.5% greater at the same applied pressure as monolayers, and this difference grew as the applied pressure increased as well (47). This may be because the hydrogel provides spheroids with an extra absorption coefficient and specific heat capacity (Table 1), which allows the latter to absorb a significantly greater amount of heat than monolayers without hydrogel but only results in a gradual rise in temperature. This could explain why, in the monolayer scenario, larger intensities resulting in higher tem-

peratures were needed to accomplish the same ablation %. (49), 50, and 51.However, compared to monolayers, the spheroids heated up substantially more to lower temperatures for the same sonication parameters, suggesting that the disruption of the microenvironment affected the survival of the tumour cells, making them more vulnerable to mechanical ablation than thermal ablation at this point (48). A sequence of cavitation events occurred as a result of residual gas bubbles inside the spheroids producing cavitating bubbles that caused mechanical damage as opposed to thermal damage at high negative pressures and shorter HIFU pulses (49), 50, and 51.

Partial and Complete Cellular Ablation in Spheroids

At low DC and sonication intensities, cells in the innermost layer of the spheroids remained unharmed, whereas those in the outermost layer were destroyed first. At the conclusion of each sonication session, complete ablation of every cell in the clusters was attained as DC and intensity were raised. The outermost cells of the cluster are more exposed to oxygen than the cells at the centre, which develop into hypoxic areas, because spheroids have a gradient in oxygen concentration from the outer to the inner core (20). As a result, when the temperature rose, the latter were more vulnerable to ablation at higher intensities. However, microscopic pictures revealed that the hypoxic centre of a cell cluster was ablated after its outer surface, indicating a gradient in the distribution of ultrasonic power per unit volume as it moved across the cells. Gradients in pH, oxygen concentration, and metabolic activity are formed in 3D cell cultures due to the more complex interconnections between the cells (52). Although the heat distribution is homogeneous throughout, these cell-to-cell contacts may create a shielding effect for the cells at the core.

Model Validation

Using an ultrasound transducer to simulate the passage of non-linear acoustic waves through different media layers is necessary to increase the efficacy and efficiency of HIFU treatment. In these simulations, several ways for focusing the waves are tried out, and it is observed how they raise the temperature of the targeted tissue and cause lesions to occur. For the purpose of organising and refining HIFU techniques intended for clinical application, these computational studies are essential. The disparity between the highest temperature attained by the two simulated cell designs was brought to light by our computational model. Because of their higher acoustic impedance—that is, the higher density of the spheroids culture media (RPMI) relative to the monolayer (DMEM)—spheroids were specifically exposed to a higher heat deposition. Additionally, the thicker layer (117 μm vs. 15 μm) that characterises spheroids generally raises their total impedance. These factors led to increased heat fixation because more acoustic energy was deposited in the cells. Even still, the spheroids' temperature was noticeably lower even though the specific heat capacities of the two culture media were comparable. The reason for this could be that the spheroids gel's conductivity (0.53 W/K.m) is 75% higher than the monolayer's (0.13 W/K.m). As a result, the temperature levels reported with the spheroids were lower due to increased heat conduction and dissipation to the surroundings. When compared to monolayers, this effect and the hydrogel medium's increased specific heat capacity cause the temperature inside the spheroids to rise more slowly.

Moreover, the inner cells survived the low DC, while the spheroids were destroyed at the boundary first. All of the cells, even the deepest ones, were destroyed when the DC increased. According to our model, there was no discernible temperature difference inside the focal zone and the spheroids' temperature was constant throughout the culture medium. Zhou et al. (53) saw similar results, with the centre of the ablated malignancy looking similar to live cells following H&E staining. These cells showed no evidence of deterioration in their nuclear chromatin and cytologic staining properties. Nevertheless, electron imaging demonstrated the presence of vacuoles in the cytoplasm of those cancer cells that appeared normal, where the cell membranes were broken down and unknown organelle structures were present. implying, rather than incomplete coagulation necrosis, an irreversible cell death with the preservation of cellular structure mediated by heat fixing (53). Furthermore, because the centre portion of the ablated tumour did not mend from the wound during HIFU treatment, it resisted disintegration. On the other hand, cancer cells in the periphery exhibited the usual traits of fatal and irreversible cell destruction, such as coagulation necrosis. Because NADH-diaphorase stain is based on the presence or lack of enzyme function rather than alterations in cellular structure, it has been demonstrated to be more objective and accurate than H&E staining in the assessment of acute cell death. Additionally, Wang et al. emphasised these observations. (54) where the centre portion, which was thermally stabilised, appeared normal and comparable to live cells with the preservation of cell structure, while the periphery had suffered deadly damage. Therefore, we need to confirm that the staining

methods we've chosen are appropriate for accurately reflecting the state of cell viability.If the staining observations are indeed accurate, then one plausible explanation for this phenomena is the use of pulsed HIFU in conjunction with mechanical ablation, rather than thermal ablation, at this particular level. Spheroids may ablate at lower temperatures than monolayers due to mechanical forces. In actuality, mechanical effects such cavitation and microstreaming predominate when employing high intensity ultrasonic waves (55). A subsequent wave of acoustic waves can burst the bubble created by pulsed HIFU, causing the tissues to liquefy and become disrupted. When pulsed HIFU beams are utilised rather than continuous HIFU, these mechanical effects become more noticeable (56), when the pulse length or burst duration is less than the amount of time required for the cells to boil, produce a bubble, and then burst (57). As a result, bubbles that form in the inner or core region may be shielded from HIFU ablation by interactions between cells and between cells and the matrix, as opposed to the outermost cells where the bubbles may be more readily depleted.

The variation in temperature reached in this 3D cell culture as opposed to the monolayer may potentially be explained by the development of microbubbles in spheroids. Furthermore, the HIFU-formed bubbles may produce a shielding layer that stops HIFU waves from penetrating the core, resulting in the waves' reflection and backscatter, which lowers the quantity of thermal energy lost in the cells (58, 59). The proposed acoustic wave propagation model fails to account for the creation of bubbles, which makes it impossible to record such acoustic wave reflection. Furthermore, compared to the more realistic 3D spheroid culture, the monolayer culture's 2D geometry and tiny thickness may limit the production of these bubbles.

Conclusion

Clinical acceptability of high intensity focused ultrasound for thermal ablation of malignant tumours is growing quickly. Determining the optimal combination of parameters to represent the most important results of ablation area, temperature rise, and tumour damage without any disease recurrence requires analysing the impact of HIFU settings on 2D and 3D models of tumour cells. Compared to monolayer cell culture creation, spheroid cell culture formation more closely resembled in vivo tumours and obtained lower temperature elevation at similar duty cycles and sound intensities. Unlike monolayers, spheroids' extracellular-cell-medium imitating core might enable the interplay of mechanical and thermal ablation as DC grew. The ultrasonic DC influenced the extent of tumour ablation; a higher DC led to a higher ablation percentage. The ablation % was not significantly affected by the duration of ultrasonic sonication. The homogeneous distribution of heat among the cultivated cells was highlighted by numerical simulations conducted for both culture arrangements. Complete spheroidal ablation occurred at high DC and spatial-peak pulse-average intensity; at the lower end, only the outermost layer was ablated. By dominating thermal ablation through cavitation, pulsed HIFU can cause mechanical ablation of cells. Due to certain restrictions, not all cell interactions with the extracellular media as they would occur in an in vivo situation were examined in this study. The sensitivity of the thermocouple that was employed and its proper placement in the focal region to avoid harming the cultured medium were key factors in temperature measurements. Furthermore, different sized spheres were present in the same petri dish, which made it difficult to examine how HIFU factors impacted spheroid dimensions. Additionally, before the cells were sonicated, we were unable to count them. However, our research highlighted HIFU's capacity to ablate both 2D and 3D cultured tumours and determined how ultrasound settings affected the percentage of ablation, the area of damage, and the temperature rise after sonication.

Conflict of interest : The study was carried out without any possible conflicts of interest, according to the authors.

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