The Cytotoxic and Genotoxic Effects of Low-Intensity Pulsed Ultrasound on *VCaP* Prostate Cancer Cells

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We investigated the cytotoxic and genotoxic effects of low-intensity pulsed ultrasound (LIPUS) on *VCaP* prostate cancer cells. An ultrasound machine was constructed using a 1 MHz ultrasound transducer and was calibrated using a piezoelectric sensor connected to an audio amplifier and digital oscilloscope to confirm the carrier frequency. *VCaP* cells were exposed to ultrasound at 0.3, 0.5, and 0.7 MHz with 3-, 6-, and 9-minute time exposures for each frequency. Cytotoxic index showed that the optimal LIPUS frequency and time exposure that significantly debilitated the *VCaP* cells is 0.7 MHz at 9 minutes. This LIPUS parameter was subsequently used for comet genotoxicity assay which resulted in comparable CometScore values with the positive control Zeocin. This *in-vitro* study demonstrated that LIPUS can be used as a possible and promising alternative treatment protocol for prostate cancer.

Key Words: low-intensity pulsed ultrasound, VCaP, prostate cancer, cytotoxicity, genotoxicity

1. INTRODUCTION

Low-intensity pulsed ultrasound (LIPUS) has been used as an alternative treatment for breast cancer (Wang *et al.*, 2013; Jia *et al.*, 2015; Carina *et al.*, 2018), colon cancer (Yumita *et al.*, 2000; Sazgarnia *et al.*, 2011), prostate cancer (Warmuth, Johansson, and Mad, 2010), among others. The frequency of ultrasound ranges from 20 kHz to several GHz. However, the therapeutic ultrasound only ranges from 0.2 – 2.0 MHz frequency (ter Haar, 2007). Moreover, there has been a variety of ultrasound applications for cancer which are covered by the review article of Wood & Seghal, (2015). Ultrasound is a sound wave that can produce mechanical and thermal effects, but these effects vary depending on intensity and frequency (ter Haar. 2007). Consequently, the determination of specific intensity and frequency for different cancer diseases is important for LIPUS to be considered for clinical studies. A key challenge, however, is to determine the optimal parameters of LIPUS suitable for pancreatic cancer. Here, we conducted cytotoxicity and genotoxicity assays to test the cell- and DNA-damaging effects of LIPUS on *VCaP* pancreatic cancer cells at different frequencies and time exposures, respectively. The results of this research work could provide baseline information on the initial assessment of LIPUS on prostate cancer for clinical trials.

2. METHODOLOGY

2.1 Cell culture

The *VCaP* prostate cancer cells (ATCC, Manassas, VA, USA) were provided by the Molecular Science Unit Laboratory (MSUL), Center for Natural Sciences and Environmental Research (CENSER) of De La Salle University, Manila. The cells were cultured in a 50 cm^2 T-flask with Dulbecco's Modified Eagle medium (DMEM, Invitrogen, MA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, MA, USA). The cells were then grown in a humidified incubator of 5% CO_2 at 37°C. After reaching 90% confluence, the monolayer cells were washed twice with 1x PBS and then detached with Trypsin-EDTA followed by viability counting via trypan blue. Corresponding 1 x 10^5 viable VCaP cells/mL were seeded into the wells of a 96-well culture plate and incubated at 37°C in 5% CO_2 overnight to allow reattachment.

2.2 Ultrasound system

The ultrasound machine was built from a 1 MHz ultrasound transducer. It is connected to an arbitrary function generator (AFG1022, Tektronix, Inc.) to program the carrier frequency as shown in Figure 1. The frequency was set to variable frequencies of 0.3 MHz, 0.5 MHz, and 0.7 MHz. Calibration of the ultrasound machine was carried out by using a piezoelectric sensor connected to an audio amplifier and mixed domain oscilloscope (MDO3SA, Tektronix, Inc.) to confirm the carrier frequency.

2.3 Ultrasound treatment and cell viability

The *VCaP* prostate cancer cells were exposed to three carrier frequencies specifically: 0.3, 0.5, and 0.7 MHz at 3, 6, and 9-minute exposure times for each frequency. *VCaP* prostate cancer cells that were not exposed to any treatment served as the negative control. PrestoBlue® viability reagent (10 µL) was carefully added to the cells and then incubated for one hour. Optical density (OD) values were recorded at 570nm from both treated and untreated control groups using a spectrophotometer microplate reader (ELx800, BioTek Instruments, Inc., VT, USA) from which cytotoxic index (CI%) values were derived.

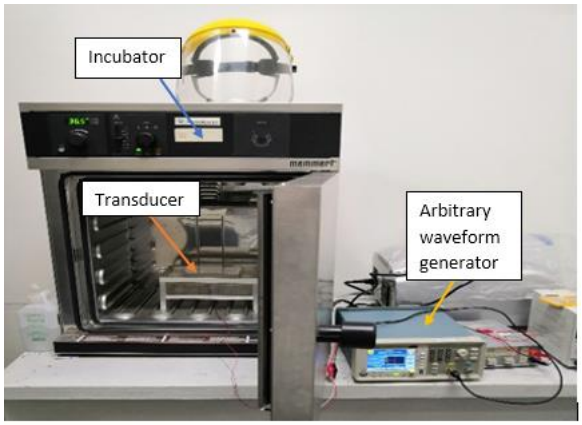

Fig. 1 Experimental setup.

2.4 Alkaline comet genotoxicity assay

The LIPUS parameter with the highest CI% was applied to assess its DNA-damaging capability in *VCaP* cells. After the LIPUS treatment, the VCaP cells were washed and trypsinized after which 50 µL of the cell suspension were carefully mixed in 100 µL of pre-melted agar. Fifty (50) µL of cell-agar suspension was carefully spread onto the comet slide using a pipette. After complete polymerization, the slides were immersed in the cold lysis solution overnight followed by alkaline unwinding for 20 min. Thereafter, the slides were subjected to electrophoresis following standard protocols. Staining was performed and fluorescence microscopy was achieved by capturing 50 photomicrographs which were later analyzed via CometScore 2.0 for tail length, % DNA, and tail moment.

2.5 Statistical analysis

Statistical analysis was used to compare data between the control and treated groups. All values are expressed as mean ± SD (standard deviation) and were analyzed using GraphPad Prism v.7.01 (GraphPad Software, Inc., USA). A p-value of < 0.05 was considered statistically significant. Moreover, Tukey's post-hoc honest significance difference (HSD) was used to further analyze for multiple comparisons after ANOVA analysis.

3. RESULTS AND DISCUSSION

3.1 Ultrasound decreases cell viability

Figure 2 presents the optical density (OD) values of untreated (n=5) and treated *VCaP* cells (n=5 per treatment). Results showed that LIPUS with carrier frequencies of 0.5 and 0.7 MHz significantly affected the viability of cells at all exposure times ($p < 0.05$). On the other hand, 0.3 MHz at all exposure times showed no significant difference compared to the untreated group ($p > 0.05$). A number of authors stress the potential therapeutic abilities of LIPUS on cancer, however, the mechanism of action is often not well understood (ter Haar, 2007).

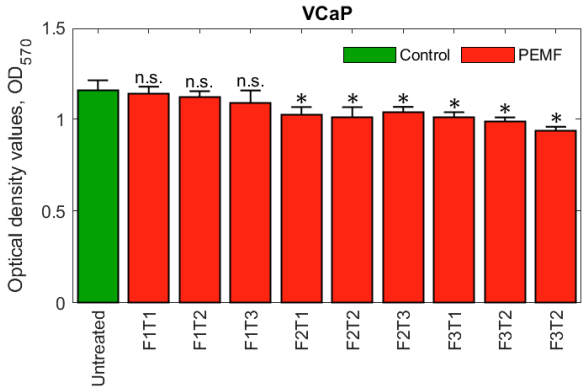

Fig. 2. The OD (570 nm) values of untreated *VCaP* (green) and treated *VCaP* (red) at different frequencies and time exposures. Asterisks (*) indicate values that significantly decreased compared to the untreated group (green); n.s. indicates no significance.
Note: F1, 0.3 MHz; F2, 0.5 MHz; and F3; 0.7 MHz. T1, 3 minutes; T2, 6 minutes; and T3, 9 minutes.

3.2 Ultrasound selectively exhibits cytotoxic effects.

Cytotoxic index (CI%) values were derived from the OD_{570} readings to quantify the degree of cytotoxic effects of LIPUS on *VCaP* cells (Figure 3). It can be observed that the percentage of dead *VCaP* cells increased as frequency increases in all-time parameters. Moreover, LIPUS frequencies of 0.5 and 0.7 MHz at all exposure times demonstrated significant VCaP cell death compared to 0.3 Mhz across all time exposures ($p < 0.05$). In this study, the most optimal LIPUS parameter was observed at 0.7 Mhz, 9 min compared among the rest ($p < 0.05$).

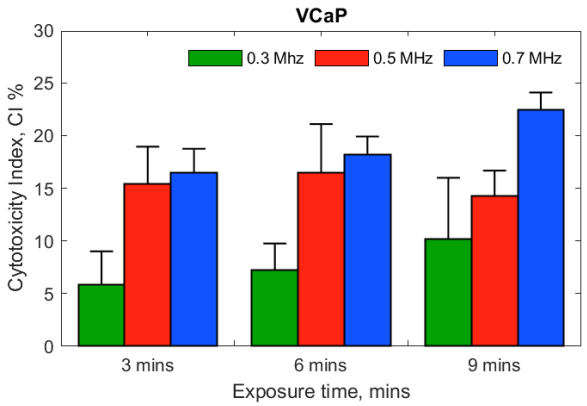

Fig. 3. Cytotoxicity index values (CI%) of *VCaP* exposed to LIPUS at 0.3 MHz (green), 0.5 MHz (red), and 0.7 MHz (blue) at different time exposures.

3.3 Ultrasound exhibits genotoxic effects

Figure 4 shows the comet fluorescence photomicrographs of *VCaP* cells after alkaline electrophoresis. It can be inferred that LIPUS treated *VCaP* cells exhibited greater CometScore parameters values (Table 1) compared to untreated control ($p < 0.05$). Moreover, the genotoxic activity of LIPUS treatment is comparable with the known antitumor selective agent Zeocin. Clearly, LIPUS treatment at 0.7 MHz can alter the DNA of *VCaP* cells.

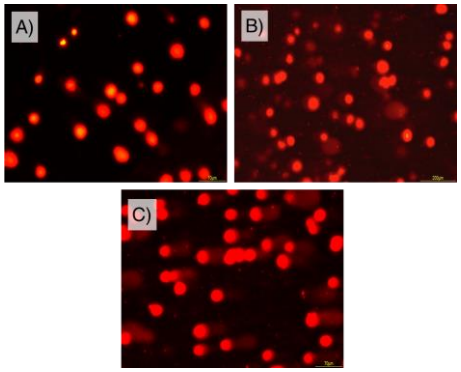

Fig. 4. Comet assay fluorescence photomicrographs of (A) untreated *VCaP* cells and (B) treated *VCaP* cells with LIPUS at 9 mins of 0.7 Mhz. Zeocin-treated cells (C) served as positive genotoxic control.

Table 1. Summary of the generated CometScores of *VCaP* exposed to different LIPUS treatments.

Treatments	CometScore Parameter		
	Tail Length	%DNA	Tail Moment
Untreated	6.83 ± 4.23	16.07 ± 4.00	3.02 ± 2.33
LIPUS at 0.7 MHz, 9 mins	24.65 ± 8.37	26.30 ± 5.54	12.04 ± 5.29
Zeocin	30.68 ± 9.14	22.99 ± 4.99	15.67 ± 6.66

4. CONCLUSION

We provided evidence that LIPUS can induce cytotoxic and genotoxic effects on *VCaP* prostate cancer cells. The experiment showed that LIPUS significantly reduced the viability of *VCaP* cells at 0.5 MHz and 0.7 MHz. Moreover, the optimal LIPUS frequency and time exposure that can debilitate *VCaP* prostate cancer cells is 0.7 MHz at 9 minutes among the applied parameters. The cytotoxic index of *VCaP* cells exposed to LIPUS demonstrated an apparent linear increase with the frequency and exposure time. The genotoxicity test showed that LIPUS can cause DNA damage in *VCaP* cells. These findings suggest the potential of LIPUS as a possible alternative treatment for prostate cancer.

5. ACKNOWLEDGMENT

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