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Terahertz Radiation Does Not Alter Nuclear Envelope of Rat Basophilic Leukemia Cells to Propidium Iodide

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ABSTRACT

Terahertz (THz) radiation technology is fast-growing, with applications in sensing, security, monitoring and pharmaceutical industries. Since it is non-invasive, THz has been used as a diagnostic and therapeutic tool in medicine, but its specific effects on biological systems are still largely under-studied. THz has been used to image tissues and cells mainly because it allows for identification of morphological features without the need for fluorescent or radioactive labels. But, the potential effects of high intensities of THz radiation are currently not well understood. One of the hypotheses that has been proposed for possible effects of THz on living cells is that it disrupts the cell membrane and induces increased permeability. To test this hypothesis, we exposed a rat basophilic leukemia cell line (RBL-2H3) to intense, single-cycle pulses of THz radiation. We then observed the internalization of propidium iodide, a fluorescent intercalating agent that binds to DNA. We did not observe any changes in RBL-2H3 fluorescence following exposure to these intense THz pulses. This observation suggests that exposure of RBL-2H3 to THz radiation may not increase their membrane permeability to propidium iodide. These experiments were preliminary, and further optimization and analysis is required before we can make definitive conclusions. However, our preliminary observations have set a baseline of RBL-2H3 fluorescent measurement in our system, have shown that it is possible to expose RBL-2H3 cells to THz radiation using our configuration, and have set the stage for future experiments.

Introduction

Terahertz (THz) radiation is non-ionizing radiation, used in diagnostic pulsed imaging and spectroscopy (Pickwell and Wallace 2006). As innovation and development of THz technology continues to progress, human exposures can be expected to increase. Understanding effects of THz on human cellular structures can help develop our knowledge of biological mechanisms of disease and may provide a foundation for further innovations and existing diagnostic and therapeutic technologies.

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Due to its low photon energy, THz radiation is unable to directly break chemical bonds and is therefore non-ionizing and non-destructive to tissue. However, external THz radiation couples to natural oscillatory dynamics of important biological structures and may influence their structure and potentially function. This unique interaction mechanism provides contrast for imaging that can discriminate diseased from healthy tissue without additional labels, thereby enhancing the potential for early detection and treatment of skin, breast, and colon cancers (Gentec 2018). THz is generally regarded as a non-destructive probe, but electromagnetic energy at THz frequencies is associated with altering molecular/cellular structural dynamics and their function. Experimentally, distinct THz absorption modes have been found in isolated DNA and protein samples (Acbas et al. 2014). These findings motivate the hypothesis that sufficiently high intensities of THz may dysregulate biological systems that rely on molecular function.

Around 155 articles have been published on the medical applications of THz. Out of those, 92 were on THz use in imaging, 20 publications were on effects of THz on cells, 26 on THz effects on tissues, and 17 on THz effects on biochemical processes (Figure 1A). Starting from 2010, 61 articles were published on the medical applications of THz (Figure 1B).

Biological effects caused by THz radiation can be classified as thermal or non-thermal. In other words, effects caused by heat, or effects due to coupling to natural oscillatory dynamics and modulating biological function before the energy is thermalized. Increase in temperature at cellular or tissue level is called hyperthermia, and this induces damage. Cells have a lipid bilayer membrane, which provides a barrier and marks the structural boundaries of a cell. When the cell is heated, it causes rearrangement of this lipid layer which increases the passages of substances into and out of the cells, called cell permeability. When a cell is exposed to stressful conditions like heat, it may disrupt the critical functions of the cell. This decreases the healthy number of cells in a given sample, also known as cell viability. THz-induced thermal effects are dependent on exposure time, therefore longer exposure leads to increased damage to biological material (Wilmink and Grundt 2011). Secondly, this could be explained by non-thermal coupling to oscillatory modes. These are the set of frequency distributions that dictate the biomolecule's natural vibrational/rotational dynamics that arise due to their unique charge/mass distributions in the cellular environment. Larger macromolecules, such as proteins, DNA, and membrane structures, have natural oscillatory dynamics that correspond to a broad range of THz frequencies. Therefore, sufficiently high intensities of THz radiation could alter the structural dynamics and biological function non-thermally (via coupling to oscillatory modes) (Fischer et al. 2012).

To test this hypothesis, we used rat basophilic leukemia (RBL-2H3) cells, an adherent rat basophil-like leukemia cell line that provides a convenient model of a live cell structure (Falcone et al. 2018). Basophils are granulocytes found at low levels in the peripheral blood (Kubo 2018). The RBL-2H3 cell line is closely related to mast cells: immune cells that act as early responders and are also involved in allergic responses. Both cell types release intracellular granules and pro-inflammatory mediators when they are activated with specific stimuli (Kubo 2018). The effect of THz on basophils is unknown, although THz exposure in skin has previously been shown to induce recruitment of immune cells known as neutrophils to the exposure site (Hwang et al. 2014). RBL-2H3 is an adherent cell line and adherence is especially important in THz research, as it minimizes the amount of water, which absorbs THz radiation, between the dish and the cell membrane (Williams et al. 2012).

The objective of our experiment was to study the biological effects of THz on mammalian cells; specifically, the internalization of propidium iodide under THz exposure and its effect on RBL-2H3 cell

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line. This study also determined the optimal cell seeding conditions for the RBL-2H3 cell line.

Considering the two possible explanations for biological effects of THz, we hypothesized that THz exposure will non-thermally induce changes in biological systems. The rationale is that non-thermal effect is achieved by using intense pulsed THz source, which delivers small average intensities (~mW/cm^2) to minimize heating. Earlier experiments by Wilmink and others showed that pulsed exposure only marginally increases temperature during all exposures, so they concluded that non-thermal mechanisms are responsible for observed morphological changes (Wilmink and Grundt 2011). Therefore, by using a train of intense pulses with a low duty cycle (~10^{-9}) to limit thermal effects, we hoped to study the non-thermal effects of THz radiation on one aspect of biological systems: membrane permeability. Current literature has supported the hypothesis that THz induces phenotypic changes on a cellular/tissue level: THz exposure has been shown to increase membrane permeability (Gallerano 2004), non-thermally induce

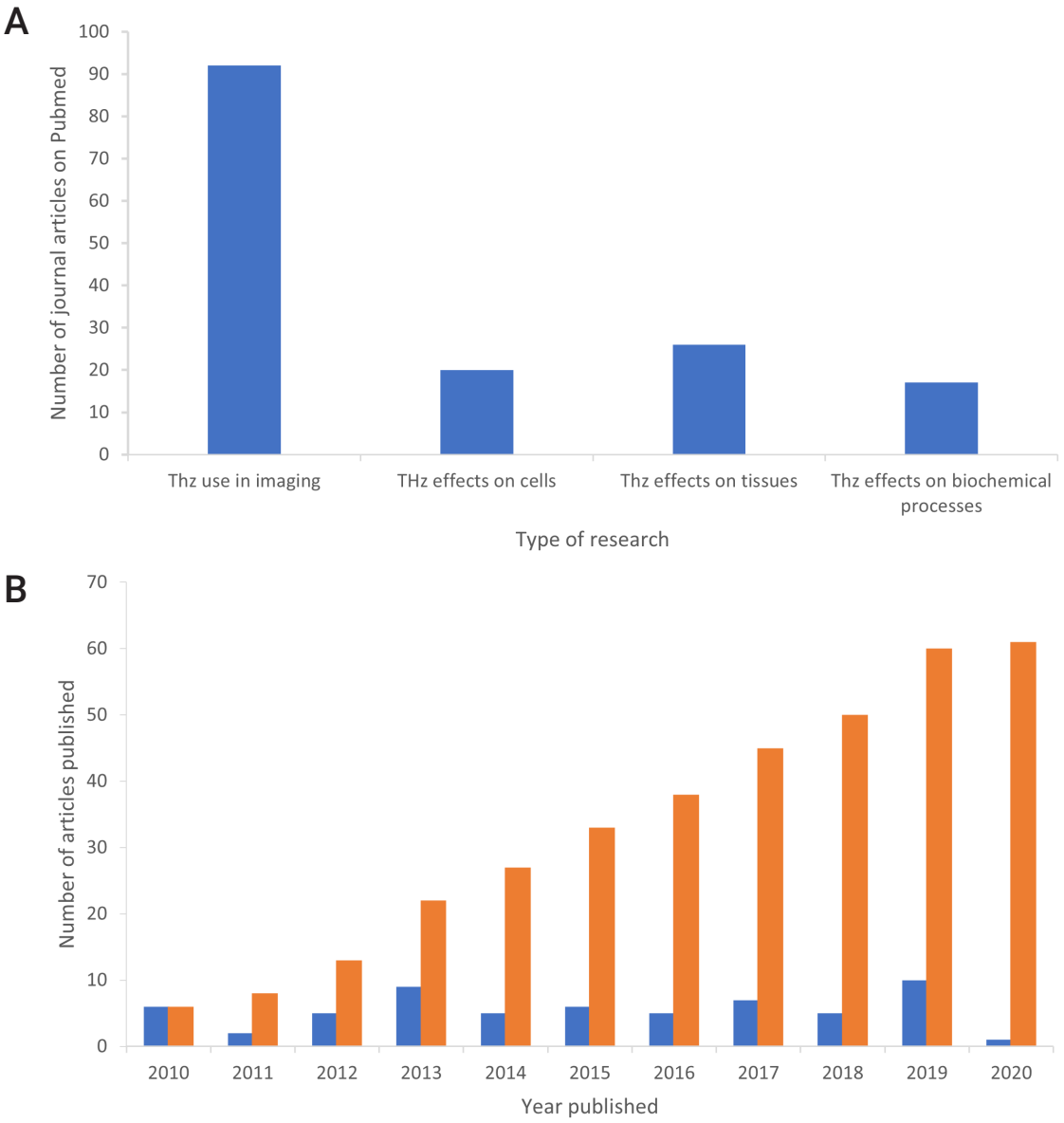

Figure 1. Research articles published on Terahertz radiation (THz) in Pubmed database. (A) Number of journal articles published on various types of THz research. (B) Number of journal articles published each year in the last decade. Blue bars indicate articles published that year while orange bars are the cumulative count of total articles to that year.

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differential gene expression in human keratinocytes and skin models and induce inflammatory-like responses (Hough et al. 2018). For this reason, we predicted that THz radiation will compromise the permeability of RBL-2H3 cell membranes.

THz has been applied as a non-destructive probe in biological systems but its effect on cell membrane permeabilization, particularly on large molecules such as propidium iodide, has not been explored. In addition, this is the first observation of THz effects on RBL-2H3 cells. Our observations contribute to the understanding of biological effects within a relatively unexplored frequency band and intensity regime. Our results may provide a foundation to enhancement of diagnostic technologies and explore possible safety issues associated with THz.

Methods

Cell splitting:

RBL-2H3 cells from the American Type Culture Collection (ATCC) were grown in Eagle's Minimum Essential Media (EMEM) (ATCC), supplemented with 15% fetal bovine serum (Fisher) in a T75 flask at 37°C in a 5% CO_2incubator. The cells were passaged every 3-4 days at 80% confluency using 0.25% Trypsin-EDTA (Fisher). Ethics approval is not required since only an animal cell line was used.

Growth of RBL-2H3:

RBl-2H3 cells were split and counted. Quadruplicate, twofold serial dilutions of cells, ranging from $1.53x10^3$ to $1x10^5$ to cells per well, were made in a 96-well tissue culture plate. Four wells were left blank as a cell free control. Cells were allowed to attach overnight, then CyQUANT™ XTT Cell Viability Assay kit (Invitrogen) reagent was added as per the manufacturer's protocol. Cells were incubated with XTT overnight and absorbance was read at 450nm, using absorbance at 660nm as a reference wavelength. Corrected absorbance at 450nm was calculated by subtracting absorbance at 660nm for a particular well from the raw absorbance at 450nm.

Propidium iodide (PI) staining:

PI is a fluorescent DNA intercalating counterstain that is used to stain dead or compromised cells. RBL-2H3 cells were cultured in a dish (ibidi µ-Dish 35 mm, low Grid-500) for several hours to allow adhesion of cells before THz exposure. Media used for seeding the cells was discarded and the cells were washed twice with Phosphate-buffered saline (PBS) (Fisher). To calibrate the fluorescent microscope, two dishes of the cells were fixed by overlaying cold 100% methanol over cells and were incubated at -20°C for 5 minutes then washed three times with PBS. To make the propidium iodide staining solution, 1 mg/mL propidium iodide stock (Invitrogen) was diluted with PBS to make 1:3000 dilution and vortexed. For each well, 0.33 µL of propidium iodide was added to 1 mL of PBS. PBS was then removed from the cells. Cells overlaid with methanol served as our controls. Methanol permeabilized cells with just the PBS served as a negative control, whereas methanol permeabilized cells overlaid with PBS+PI served as a positive control. The cells were incubated for 5 minutes at room temperature, washed twice in PBS and covered until imaging. For our experimental groups, live cells were washed twice in PBS and then they were overlaid with PBS +PI solution before THz exposure.

THz exposure post propidium iodide staining:

THz was generated by optical rectification of intense infrared laser pulses in lithium niobite at a 1 kHz repetition rate (i.e., one THz pulse per millisecond) and focused with gold parabolic mirrors to a circular spot size approximately 1.5 mm diameter. At the focus, the THz intensities were 17.54 MW/cm^2 (peak) and 24.36 mW/cm^2 (average). The pulse duration was 1.39 ps. The peak of the THz spectrum was 0.53 THz, with a full width at half maximum of approximately 1 THz.

Fluorescence imaging:

For imaging, the excitation laser had a wavelength of 473 nm. Samples that were to be exposed to THz radiation were placed on the laser apparatus and an image at time point zero was taken on

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fluorescence laser. With the THz radiation off, a 1-minute video using fluorescence laser was taken and then images were taken every 2 minutes until the 10-minute time point. At the 10-minute point, the THz beam was turned on and a 1-minute video was taken. After this, images were taken every 2 minutes until the 20-minute point was reached. After exposure, the cells were imaged again using an Olympus IX81 fluorescence microscope at 20X magnification. As a control, another area of the dish distant from the small region exposed to the focused THz beam was imaged. When finished, the cells were discarded in autoclave waste.

Results

RBL-2H3 cells grow:

To ensure RBL-2H3 cells were viable, and to determine what number of cells would be required for future cell viability assays, we utilized an XTT assay (Figure 2). This assay uses a substrate, XTT, that changes color to orange after accepting an electron indirectly from NAPDH, a major electron carrier used in anabolic reactions within the cell. The amount of orange color produced is correlated with the metabolic activity of the cell. When the cells were seeded at a density between 0 cells to $1.25x10^4$ cells, the

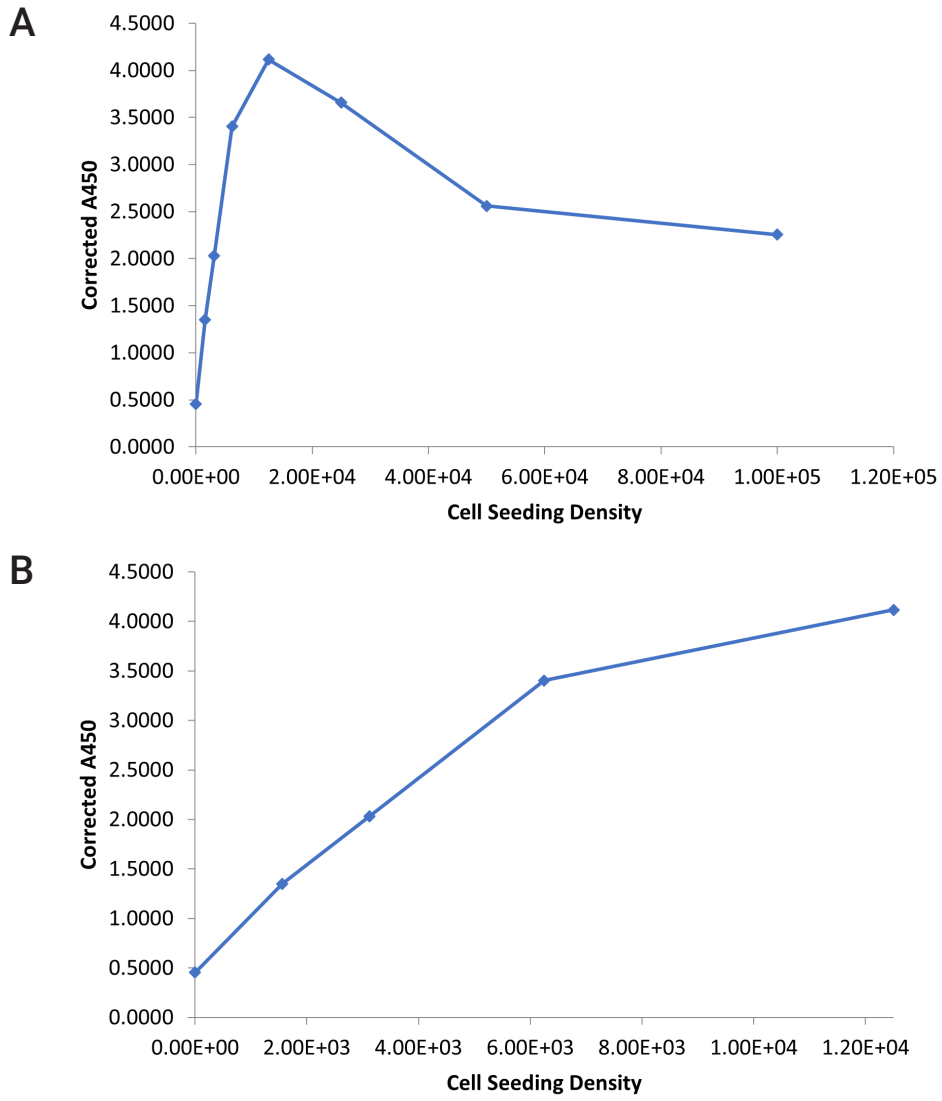

Figure 2. Optimization of cell seed density for XTT assay. (A) Standard curve was made from dilution of cells from 0 cells per well to 105 cells per well to correlate the density with absorbance signal. (B) The linear part of standardization curve was taken for the optimal cell density.

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absorbance goes up from 0.4546 to 3.4035. At the cell density of $2.5x10^4$ cells, the absorbance decreases from 3.4035 to 4.1157. When the cell density is $1.00x10^4$ cells, the absorbance decreases to a lower value of 2.2533 (Figure 2A). This graph shows that cells grew optimally when seeded at or below a density of $1.25x10^4$ cells. Higher concentrations are inadvisable for future experiments, as cells are likely too dense, and growth is inhibited.

THz exposure does not appear to increase propidium iodide internalization:

The effect of staining RBL-2H3 cell lines before and after exposure to THz radiation was shown (Figure 3). RBL-2H3 cells overlaid with methanol, which permeabilizes cell membranes, serves as a positive control (Figure 3A). RBL-2H3 cells were visualized under a fluorescence laser when the THz had been turned off (Figure 3B). Since the cells were left in the apparatus in the absence of a THz beam, this serves as our negative control. When cells prior to THz exposure were visualized under a fluorescent microscope (Figure 3B), a small, bright circle appeared. This was a dead cell that picked up propidium iodide before exposure to THz, and hence it is fluorescent. When cells were visualized in the absence of THz radiation, no change in fluorescence was observed after 4, 6, or 10 minutes of exposure (Figure 3B i-iv). Figure 3B (iii) and (iv) look bright because luminance was increased solely on the basis of better visualization. Over

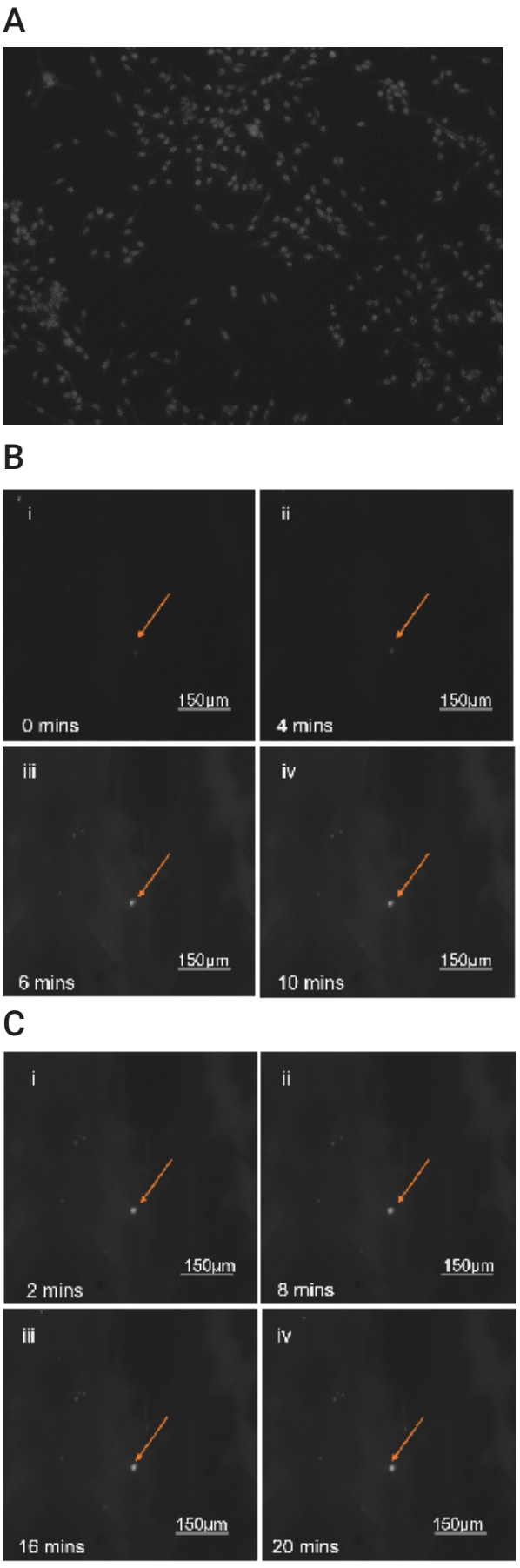

Figure 3 Effect of propidium iodide staining before and after Terahertz exposure on RBL-2H3 cell lines. (A) Methanol treatment permeabilized cell membranes and hence fluoresces when stained with propidium iodide (B) RBL-2H3 cells were visualized under fluorescence laser for 10 minutes when the beam was turned off. These unexposed cells were imaged every 2 minutes for a total span of 10 minutes. (C) Images were taken every 2 minutes for a total of 20 minute (i) 2 minutes after exposure (ii) 8 minutes after exposure (iii) 16 minutes after exposure and (iv) 20 minutes after exposure. The arrow indicates a dead cell that had detached before the imaging began.

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10 minutes, there was no increased fluorescence since the apparatus had constant conditions prior to THz exposure.

Propidium iodide stained RBL-2H3 cells were then exposed to a THz beam for 20 minutes, and images were captured (Figure 3C). Upon exposure of cells to THz radiation, no change in fluorescence was observed after 2, 8, 16, or 20 minutes (Figure 3C i-iv). No new cells picked up propidium iodide and fluorescence had not increased. This indicates that post THz exposure did not increase membrane permeability to a significant enough degree to be detected by microscope.

Discussion

Although profound phenotypic changes have been observed following THz exposure (Gallerano 2004), we hypothesized that more subtle changes in cell membrane dynamics can also occur. THz radiation non-thermally induces significant phenotypic changes in cellular systems because THz is coupled to natural oscillatory dynamics of biological systems, rather than merely heating. Experimentally, this is achieved by using an intense pulsed THz source with 1 kHz repetition rate (1 pulse per ms). This allows for large peak pulse intensities (~MW/cm^2) to be delivered to overcome the large attenuation in aqueous environments, maintaining small average intensities (~mW/cm^2) to minimize heating. We tested this hypothesis using rat basophilic leukemia (RBL-2H3) cells, which provide a convenient model system for monitoring changes in cell behaviour. RBL-2H3 cells were used since they are relatively easy to work with and our lab has expertise in working with these particular cells. They are also adherent, unlike a lot of other leukocytic cell lines, which is necessary to reduce the amount of water between our plate and the cell membrane, as water would reduce the intensity of the incoming THz radiation (Williams et al. 2012). It is possible that different cell types, with different membrane compositions, could be more or less sensitive to permeabilization by THz.

To determine whether there were changes in the cell membrane of RBL-2H3, we used a common "cell-permeability dye" called propidium iodide. Propidium iodide (3,8-Diamino-5-(3-diethylamino-propyl)-6-phenylphenanthridinium iodide methiodide) is an intercalating molecule that binds DNA with no particular sequence preference and it only fluoresces once bound to DNA (Rosenberg et al. 2019). Propidium iodide has an excitation maximum of 493 nm and an emission maximum of 636 nm (Emerson et al. 2017). Since propidium iodide is not membrane permeable, fluorescence indicates internalization through a compromised lipid membrane into the nuclear envelope. For this reason, propidium iodide is useful in distinguishing apoptotic or necrotic cells within a population of healthy cells. Propidium iodide is used to assess cell permeability, and it is used in assessing the intactness of cell membranes. Since THz exposure did not cause any significant changes in fluorescence, (Figure 3) our prediction that THz would increase permeability was not confirmed.

Our qualitative data showed no detectable visual changes in propidium iodide fluorescence in the RBL-2H3 cells following exposure to THz, suggesting that there were no significant changes in cell membrane permeability. Only qualitative experimental data was collected because, as seen in the methanol permeabilization pictures (Figure 3A), the increase in fluorescence if the cell membranes are substantially permeabilized can be sufficiently identified qualitatively. Additionally, to increase the credibility of our results, multiple trials should be performed. Since the results were negative, we had planned on trying various other strategies to see if we could detect more subtle types of membrane permeabilization. Due to the difficulty in setting up the laser apparatus, we wanted to try the most informative strategy. We were unable to replicate our results due to COVID-19 pandemic and shutting down of laboratories. However, these observations are preliminary and further experimentation is needed.

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Unless high intensities of THz stimulation are used, it might be hard to detect sensitive changes in plasma membrane permeability. Ribonucleic acid (RNA) is located in the cytoplasm of the cell. Therefore, using a dye that has higher affinity to RNA might help in detecting subtle changes caused due to THz. SYTO RNASelect green fluorescent stain selectively stains RNA, and it exhibits bright green fluorescence when it binds to RNA (Thermo Fisher). Using an RNA dye expands the possibility of noticing increases in cell permeability post THz exposure, since it would not have to travel through the two additional membranes (nuclear membranes), and fluorescence can be observed if the dye passes through the plasma membrane.

According to a recent study by Vernier and others, lipid pore formation occurs with THz exposure, but the changes might be too small to be detected. For any applied electric field over a certain time interval, there is a probability for rearrangement of lipid molecules in the bilayer. With increase in the electric field, the probability of membrane pore formation increases exponentially. The pores detected in the observational study of this window only represents a fraction (10^{-7}) of the area of a typical cell membrane (Vernier et al. 2015). Therefore, it is possible that the electric field we applied rearranged the lipid bilayer membrane of the cell and induced pores in the membrane. However, these changes might have been too small to be detected. In our experiment, we used a pulse length of 1.39 ps, and this article reports that water dipoles in the interior of these model membranes respond in less than 1 ps by reorienting at THz frequencies. They align in the direction of the electric fields, thus breaching the normal barrier function of the cell. Since these pores are very small, it is likely that propidium iodide could not pass through the membrane, explaining the reason behind no significant observable differences in cell permeability (Figure 3).

Additionally, the average intensity of 24.36 mW/cm^2 may not have been sufficiently high to create the pores that were modelled by the study by Vernier and others. Since the THz beam did not seem to be powerful enough to affect cell permeability, it may be beneficial to consider other parameters. For example, altering spectral content, the repetition rate (time between the pulses), or increasing the length of THz exposure on RBL cells may potentiate differences in cell permeability post THz exposure.

When mammalian stem cells were exposed to THz at an average intensity of ~1 mW/cm^2 for 2-6 hours, there were no observed changes in cell viability (Bock et al. 2010). However, the authors of this study proposed that it might be interesting to observe longer term growth of cell cultures after exposure. In a study by Alexandrov and others, human cells were exposed to continuous wave radiation at 227 mW/cm^2 for 1-40 minutes. This study analyzed that the vast majority of the cells exposed to this kind of THz radiation for more than 20 minutes appeared to be undertaking apoptosis, a pathway in which the cells sense stress and undergo programmed cell death (Alexandrov et al. 2011). The same study reports that when the peak intensity of THz is reduced to 84.8 mW/cm^2 for up to 80 minutes, 90% of THz exposed cells survived these conditions with minor increases in heat shock proteins.

A study by Giles and others showed that *Bacillus subtilis* bacterium was irradiated with THz for 1, 2, and 24 hours with an intensity of 1.3 mW/cm^2. They used XTT assay to measure cell viability and they found that the optical density between experimental wells exposed to THz and the control wells was found to be insignificant (Giles et al. 2012). Since THz intensity of 1-1.3 mW/cm^2 was insignificant, and peak intensity of 84.8 mW/cm^2 induced thermal effects by increasing heat shock proteins, we suggest that future studies optimize the THz parameters within this range. According to occupational safety standards of THz, 5-50 mW/cm^2 is considered safe, and for general public exposure, 1-10 mW/cm^2 is considered safe, when the frequency is less than 0.3 THz (Giles et al. 2012). We used a power density within the range of occupational safety

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standards for our experiment (24.36 mW/cm^2) to contribute to the understanding of THz effects on viability. This range of THz parameters is also likely to be applicable in clinical settings. Cecil and others showed that imaging of nonmelanoma skin cancers required a power incidence range of 7-25 mW (Cecil et al. 2011). Furthermore, for clinical imaging using THz, the safe average power density exposure is set to 10 mW/cm, and the average power used for imaging is 8 mW (Taylor et al. 2011). Both of these values are well within the range of occupational safety standards and therefore comparable to clinical applications. Therefore, it might be useful to explore within the occupational safety standards to further explore biological safety standards of THz.

Future Directions

For future experiments, robust quantitative data involving key parameters of THz, like pulse duration, frequency, and length of exposure, should be considered. Additionally, multiple trials must be performed to further develop our hypothesis. We hope to probe the effects of THz radiation on RBL-2H3 cell viability using an XTT assay, having shown which seeding concentrations are suitable for future experiments (Figure 2). We can imitate in vivo conditions and use temperature- and gas-controlled THz apparatus to understand biological safety levels. Biological effects of other cell lines, like glial cells, embryonic cells, stem cells, or human fibroblasts, can be explored to identify effects of THz. We can probe for molecular dynamics, including the behavior of DNA, and experiment whether THz has an effect on DNA structure or function. This can be done by assaying for any changes in expression of proteins associated with DNA damage or repair. For instance, Titova and others exposed skin to intense THz pulses and detected an increase in expression of proteins involved in DNA damage repair (Titova et al. 2013)

Research on THz radiation has only recently begun, but progress in medical, military, and security applications has served the need to enhance our fundamental understanding of mechanisms of THz that govern interactions with biological systems. Considering increasing applications of THz in biomedical fields, it is crucial to evaluate potential health hazards and develop biomarkers for detection of THz radiation exposure, and also look into potential applications for cancer therapy and wound therapy. In summary, our data suggests that no significant changes in membrane permeability are seen due to THz exposure.

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