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RESEARCH ARTICLE

The Effects of Non-Ionizing Radiation (NIR) Towards Cytoplasmic Activity and DNA Integrity in Acanthamoeba sp. Paradigmatic

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Abstract Electromagnetic fields (EMFs) with low frequency, usually used in communication (known as radiofrequency, 30kHz-300GHz) and generated by electricity (called extremely low-frequency, 3Hz-3kHz), known as the non-ionizing radiation (NIR) which lacks energy to induce ionization directly. In the current situation, the high exposure of NIR has been used for communication purposes. Wi-Fi is a wireless network that consists of at least one antenna, providing access to the internet and/or other wireless devices, such as laptops, computers, and mobile phones, which communicate wirelessly using Wi-Fi. The Wi-Fi produces different strengths of frequencies (3G, 4G and 5G). *Acanthamoeba* sp. is classified as an eukaryotic organism which is like human cells and acts as a good bioindicator for other eukaryotic organisms. Their morphological changes can be easily detected whether in healthy or unhealthy conditions. This study aims to investigate the effects of three different strengths of Wi-Fi frequency (3G, 4G and 5G) on the morphological changes in *Acanthamoeba* sp. The percentage of cell viability was observed by undergoing MTT assay technique. It was shown that the higher the frequency of NIR, 5G – the lower the cell viability counted. Light microscopy was used to observe morphological changes of the cell and cytoplasmic activity, under fluorescence microscopy and stain cells with the Acridine Orange and Propidium Iodide (AO/PI) dyes. Based on that, the frequencies of NIR are directly proportional to the cell death, autophagy and necrosis of *Acanthamoeba* sp. Precisely, the cell with treatment of 5G frequency of NIR showed the highest number of necrotic cells compared to 3G and 4G frequencies. Treatment using NIR frequencies has the potential to affect cellular health, and its presence in the environment is detrimental to living organisms. DNA integrity was observed by gel electrophoresis systems to determine the DNA fragmentation occurred after exposure to different strengths. DNA fragments showed that late autophagy and necrosis modes of cell death in cell.

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Introduction

Electromagnetic fields (EMFs) are bundles of energy with no mass, and they differ in terms of frequency and wavelength. Those EMFs with low frequency, usually used in communication (known as radiofrequency, 30kHz-300GHz) and generated by electricity (called extremely low-frequency, 3Hz-3kHz). They are known as the non- ionizing radiation (NIR), which lacks energy to induce ionization directly. However, when these EMFs are sufficiently strong, they may cause tissue heating, which is

essential for microwave oven functioning [1].

The exposure of frequency used in our daily life has been confirmed to be under the maximum limit and is safe to be used [2]. NIR has various applications in our daily lives, including in hospitals (ultrasound, x-rays), households (microwave, mobile phone, television) and industries (packing food, smoke detector) [3]. Some of the advantages of NIR are the widespread use of wireless technology widely spread globally, make it easier for the medical field to get results and do treatment in a short time and is used for sterilization tools. On the other hand, it comes with a few disadvantages, which are tissue heating (thermal effects), disturbing cells' function for too long exposure and leading to a long-term of cancer risk.

In the current situation, the high exposure of NIR has been used for communication purposes. Wi-Fi is a wireless network that consists of at least one antenna, providing access to the internet and/or other wireless devices, such as laptops, computers, and mobile phones, which communicate wirelessly using Wi-Fi. As a result, each similar wireless communication device lets wirelessly communicate using internet [4]. Wi-Fi usage has become essential in today's modern world, and the negative effects or consequences for biological systems are unknown. The Wi-Fi produces different strengths of frequency (3G, 4G and 5G).

Acanthamoeba sp. is a genus of a free-living amoeba and it was discovered over nine decades ago. *Acanthamoeba* sp. can be found all over the world and may be found in a variety of environments, including air, soil, and water [5]. The *Acanthamoeba* sp. life cycle is divided into two stages: reproductive trophozoites and dormant cyst forms. They have been classified into three different morphological groups according to their morphological characteristics: group I (well-separated ectocyst and stellate endocyst), group II (wrinkled ectocyst and polymorphic endocyst and arms) and group III (smooth ectocyst and round endocyst with no arms) [6].

Cyst formation occurs by a process called encystation. It commonly happens when *Acanthamoeba* sp. in trophozoite form was exposed to extreme conditions such as changes of pH, lacking amount of nutrients or getting treated with therapeutic agents [7]. The trophozoite form appeared in a favorable condition for the *Acanthamoeba* sp such as optimum pH, temperature, and enough source of nutrients for it to grow. It is known as an active and reproductive stage and the surface is covered with cilia [8].

Acanthamoeba sp is classified as a eukaryotic organism which is like human cells and act as a good bioindicator for other eukaryotic organisms. Their morphological changes can be easily detected whether in healthy or unhealthy conditions. They can even grow perfectly in room temperature without the CO_2 supply [9]. The 5G strength of Wi-Fi is the newest generation of frequency for the wireless connection and has the strongest frequency compared to 3G and 4G frequency. Thus, this study aims to investigate on the effects of three different strengths of Wi-Fi frequency (3G, 4G and 5G) on the morphological changes in *Acanthamoeba* sp. The observed cellular damage demonstrates the mechanism of cell death when exposed to Wi-Fi at various frequencies. This research is more feasible using Acanthamoeba because the exposure can be conducted outdoors near available Wi-Fi sources.

Based on previous studies, ionizing-radiation has been studied extensively to determine the mode of cell death on *Acanthamoeba* sp [1]. However, there is insufficient evidence regarding non-ionizing radiation (NIR) that can induce the mode of cell death of *Acanthamoeba* sp. Thus, the different strengths of NIR frequency (3G, 4G and 5G) have been exposed to *Acanthamoeba* sp. cells to identify the mode of cell death and morphological changes detected by few different techniques.

It is crucial to investigate the effects of non-ionizing radiation (NIR) at various frequencies (3G, 4G, and 5G) on *Acanthamoeba* sp. as it provides information on the effect of NIR in eukaryotic organisms, *Acanthamoeba*sp. From this study, morphological changes on *Acanthamoeba* sp. were detected by the mechanism of the mode of cell death. NIR with different strengths exhibit different percentages of cell viability on *Acanthamoeba* sp.

To ensure the mechanism of *Acanthamoeba* sp cell death, cellular and molecular analysis was conducted. The scope of this project is to focus on the effects of non- ionizing radiation (NIR) with different frequencies (3G, 4G and 5G) on *Acanthamoeba* sp. Firstly, the determination percentage of cell viability of *Acanthamoeba* sp. cells upon being exposed to 3G, 4G and 5G frequency. The percentage was determined by assessing the viability of the cells using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay technique. Next, observation of morphological changes on *Acanthamoeba* sp. by observing cells under the inverted light microscope. The determination of the mode

of cell death was separated into two where the apoptosis cell death was detected via DNA laddering technique, where the DNA fragmentation was is observed by the electrophoresis system. In contrast, necrotic and autophagy cell death was detected by using the AO/PI staining. Then it was observed under the fluorescence microscope in the dark room. Therefore, the objectives of this study are to determine the NIR strength in inducing cell death of *Acanthamoeba* sp. and observe the changes in *Acanthamoeba* sp. when exposed to NIR as well as determination of DNA integrity for mode of cell death confirmation.

Materials and Methods

Culture media preparation

Preparation of Acanthamoeba Page's Amoeba Saline (PAS) solution

Page's Amoeba Saline (PAS) was prepared in two stocks. Stock 1 was prepared by adding 0.24 g of NaCl, 8 mg of $MgSO_4$, 8 mg of $CaCl_2$ in a beaker with distilled water until reach the final volume, 1 L. As for stock 2, it was prepared on different beaker with 0.284 g of Na_2HPO_4 and 0.272 g of KH_2PO_2 was added along with distilled water until reach the final volume, 1 L. Both stocks were autoclaved at 121°C for 15 minutes and then stored in the refrigerator for further use.

Preparation of peptone-yeast extract-glucose (PYG) media solution

Peptone-yeast extract-glucose was prepared in a blue cap bottle with 7.5 g of yeast extract, 7.5 g of protease, 16.5 g of D (-) monoglucose, and 5 mL of PAS solution of both stock 1 and stock 2. Distilled water was added in the blue cap bottle until reached the final volume (1 L) and autoclaved at 121°C for 15 minutes. Lastly, stored the solution in the refrigerator.

Cultivation and Maintenance of *Acanthamoeba* sp.

*Acanthamoeba*sp. cells ((isolated from Hospital Kuala Lumpur) was subculture into the new T-25 culture flask after two to three days with 20 mL PYG media. Before exposing it to the non-ionizing radiation treatment, the cells were allowed to grow to 80% confluency in the incubator for two to three days. The cell was observed under the inverted microscope to check their confluency.

Cell Counting of *Acanthamoeba* sp.

Acanthamoeba sp. cells attached to the culture flask were brought to suspension by gently flushing them using a sterile disposable dropper. The cells then poured into a centrifuge tube (15 mL vial). Then, the tube was centrifuged for 15 minutes at 3000 rpm to get the pallet of cells before the old media was removed from the tube completely. New PYG media (13 mL) was added in the tube and flushed gently for the cells to be mixed. Cells from the tube were taken for 100 µL and added into each well. The cell number was measured by using the haemocytometer, then observed under the upright microscope and cell counting was done. The plate was incubated at 30°C for 24 hours. To obtain the concentration needed for each well, the volume of cell suspension taken from this culture was calculated using the formula below:

Cell count = Average of cell count from all squares x Dilution Factor (D. F.) x 10^4

Determination of The Cell Viability of *Acanthamoeba* sp. Cells by MTT Assay Technique

After incubation as mentioned in section 3.3, the treatment step started by exposing the plate inside a Faraday's cage with different frequency (3G, 4G and 5G) on separate days by using a vector spectrum analyzer (Keysight N9913A, USA) respectively. Each frequency was exposed to the cells for 24 hours. The well plate was taken out from the cage and MTT solution (20 µL) was added in each well. The plate was incubated for 4 hours. After incubation, DMSO (150 µL) was added, and plate incubated for another 5 minutes. The absorbance reading was read by the ELISA reader at 570nm wavelength. The value shown was used to plot a graph of cell viability against the different strength of NIR frequency (3G, 4G and 5G).

Observation of Morphological Changes in *Acanthamoeba* sp.

under Inverted Microscope

Acanthamoeba sp. cells (1 x 10^4 cells/mL) in a 6-well plate was treated with the different frequency on different day in Faraday's cage for 24 hours respectively. The morphological changes were assessed under the inverted light microscope.

Determination of Mode of Cell Death in *Acanthamoeba* sp. for Cytoplasmic Activities

Fluorescence microscope by using AO/PI Staining

Acanthamoeba sp. cells was treated following the section 3.5. The *Acanthamoeba* sp. cells in the plate was harvested. A few drops of AO/PI dye were added into each well of plate and left for 10 minutes in the dark room as AO/PI dye is sensitive to the light. The 6-well plate then observed in a dark room by using the fluorescence microscope using a blue filter and the mode of cell death of the *Acanthamoeba* sp. cells was identified.

DNA Laddering Technique

Acanthamoeba sp. cells were treated with different treatments in different 6-well plate. The first plate with *Acanthamoeba* sp. cells (1 x 10^4 cells/mL) was exposed to different frequency on different day respectively, in Faraday's cage for 24 hours. Another plate acted as the positive control and treated with the hydrogen peroxide (H_2O_2). After treatment, DNA extraction was done by following the manufacturer's protocol, InnuPREP DNA Mini Kit from ANALYTIK JENA. 20 µL of the extraction was inserted in the agarose gel for the electrophoresis system at 20 mV for 4 hours. After 4 hours, Syber Safe staining was added to add the stain on cell's DNA. It was observed by using the Gel Documentation System and DNA 'ladder pattern' were obtained.

Results and Discussion

Determination of Cell Viability Percentage (%) of *Acanthamoeba* sp. upon Exposed to Non-ionizing Radiation (NIR) by MTT Assay

Acanthamoeba sp. was exposed to different frequencies of NIR, which are 3G, 4G and 5G in three different 96-well plates for 24 hours on different days respectively. This was done to determine the effect of radiation on *Acanthamoeba* sp. This assay was carried out to assess the percentage of cell viability of *Acanthamoeba* sp. after being exposed to the different strength of NIR. Figure1 shows the graph percentage of cell viability of *Acanthamoeba* sp. against different strengths of NIR. Based on the graph, the highest frequency of NIR (5G) showed the lowest percentage of cell viability after the exposure compared to 3G and 4G. This indicates that all different strengths of NIR showed a reduction on the percentage of cell viability of *Acanthamoeba* sp. upon the exposure.

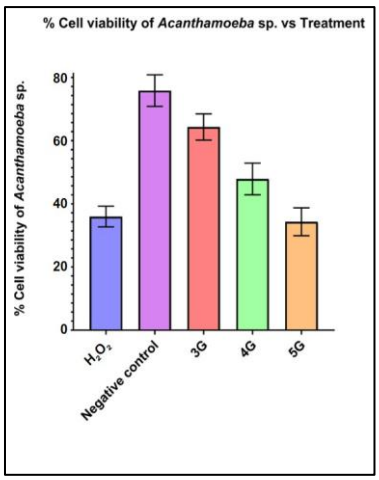

Figure 1. Percentage of cell viability of *Acanthamoeba* sp. after exposure to different NIR strength (3G, 4G and 5G) for 24 hours and assessed by MTT assay.

The MTT assay reagent, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide), is a monomeric tetrazolium salt characterized by a quaternary tetrazole ring core. This core comprises four nitrogen atoms enclosed by three aromatic rings, consisting of two phenyl moieties and one thiazolyl ring. Reduction of MTT leads to the disruption of the central tetrazole ring, resulting in the creation of a violet-blue water-insoluble compound called formazan. Due to its positive charge and lipophilic structure, the MTT assay reagent can pass through both the cell membrane and the mitochondrial inner membrane of viable cells. In metabolically active cells, it undergoes reduction to formazan [10].

The *Acanthamoeba* sp. cell was exposed to three different strengths of non-ionizing radiation (NIR) – 3G, 4G and 5G, for 24 hours on separate days respectively in three different 96-well plates. This is to assess the decrease of percentage of cell viability of *Acanthamoeba* sp. From Figure1 , the percentage of cell viability was declining as the strength of NIR increased. This shows that the viable cell in the plate was adversely affected upon exposure to NIR. The lowest percentage of cell viability was obtained by 5G frequency (33%) and followed by 4G with 50% cell viability. According to the band of wave produced by 5G frequency, it is the highest with 40 GHz and considered as the strongest among the other two frequencies [11].

Morphological Changes of *Acanthamoeba* sp. under Light Inverted Microscope

Acanthamoeba sp. was observed under a light inverted microscope after 24 hours being exposed to different NIR strength (3G, 4G and 5G) on different days respectively in three different 6-well plates. Figure 2 shows the comparison of shape of *Acanthamoeba* sp. between the untreated cells where its acanthopodia can be clearly seen in healthy untreated *Acanthamoeba* sp. cell (A) and treated with the hydrogen peroxide (H_2O_2) as positive control that showed a completely round shape indicate cyst form (B). *Acanthamoeba* sp. that exposed to different NIR, 3G (C) and 4G (D) showed that cells started to shorten their acanthopodia and enlargement of vacuoles start to form within the cell. As for *Acanthamoeba* sp. cells exposed to 5G (E) appeared in a complete cyst form.

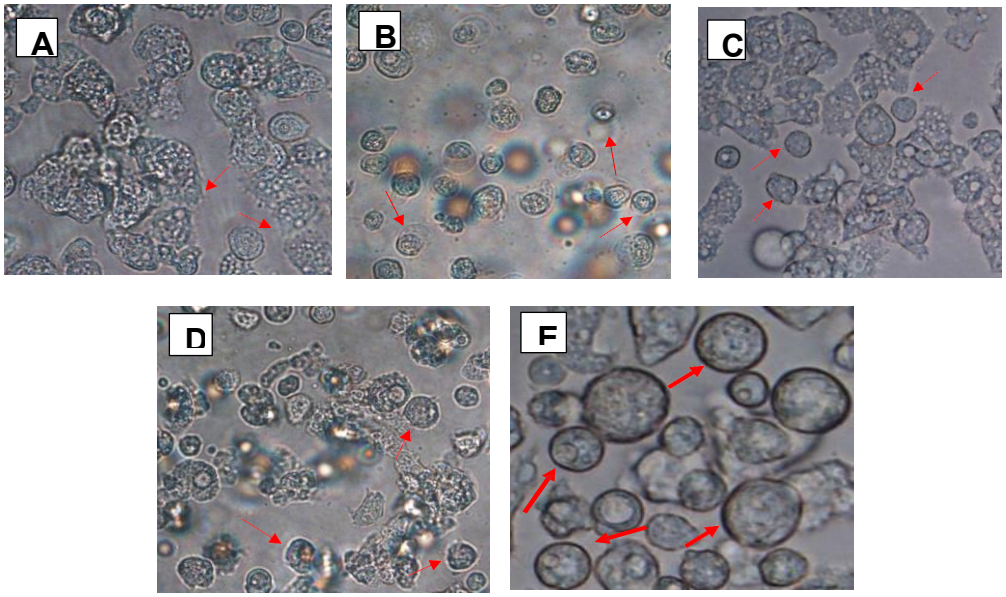

Figure 2. Images of *Acanthamoeba* sp. observed under light inverted microscope. (A) was image of untreated *Acanthamoeba* sp. with its long acanthopodia, image of (B) *Acanthamoeba* sp. cell in cyst form after treated with hydrogen peroxide (H_2O_2). (C) and (D) show that *Acanthamoeba* sp. cells starting to become rounded shape and undergoes vacuole enlargement after exposed with 3G and 4G, respectively. *Acanthamoeba* sp. cells treated with 5G (E) appeared in a complete single-layer cyst form after exposure (Magnification: 400x).

The untreated (negative control) and treated of *Acanthamoeba* sp. were observed under the inverted light microscope to observe any changes of cell morphology after the exposure. Based on Figure 2 (A), it shows that the untreated cells' shape is in trophozoite form with long acanthapodia. As no radiation is exposed to cells, it can maintain their shape that is used to attach to the surface of culture flask and for feeding. As in Figure 2 (B) and (E), cells have completely changed into cyst form due to treatment with hydrogen peroxide, H_2O_2 (positive control). Cyst form indicates that cells undergo encystation process – morphology changes from irregular shape to round shape and lose its acanthapodia. This form is inactive and resistant with double double-walled membrane [12].

In Figure 2 (C) and (D), it showed that cells slowly lose their acanthapodia and become rounded shape as they exposed to 3G and 4G frequencies respectively. The encystation process is happening, and incomplete compared to Figure 2 (E). Given that treated cells did face morphological changes, it clarified that the exposure to NIR frequencies has caused crucial inhibition effects toward the active stage of *Acanthamoeba* sp. The encystation process is triggered by stressful conditions, prompting the transformation of the cell from a trophozoite to a cyst. This transition occurs when antibodies selectively attach to the trophozoite cell membrane protein, forming a ligand-receptor interaction that initiates significant cellular alterations. The binding of the ligand to the receptor induces conformational changes in the receptor, forming the basis for signal transmission [13].

Determination Mode of Cell Death in *Acanthamoeba* sp.

Fluorescence Microscope using AO/PI Staining

Acanthamoeba sp. cells were exposed to different NIR strength (3G, 4G and 5G) for 24 hours in three different 6-well plate on different days respectively in determining the mode of cell death by using AO/PI solution staining. Both AO and PI gave different coloration on *Acanthamoeba* sp. that indicate mode of cell death occurred. Figure 3 shows the results obtained from AO/PI solution staining that reveal *Acanthamoeba* sp. cell with green coloration (A) of cytoplasm by AO dyes that stain DNA in nucleus with green colour and indicate all cell viable, whereas cell treated with hydrogen peroxide, H_2O_2 (B) and exposed to 5G frequency (E), showed orange colouration resulted from PI dye that stained whole cell with orange colour representing cell death that occurred which is necrosis and indicate all cells was non-viable. *Acanthamoeba* sp. cell that exposed to 3G (C) and 4G (D) showed yellow-orange coloration resulted from PI dyes stain nucleus with orange colour and represent autophagic cell death occurred and cells are non-viable.

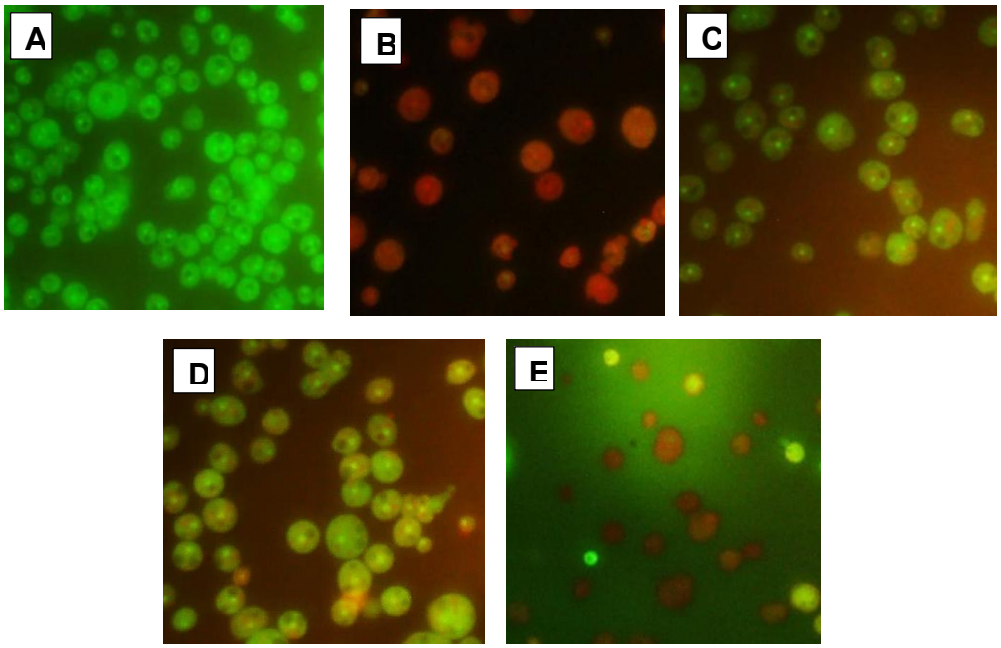

Figure 3. *Acanthamoeba* sp. cell appeared in different colouration using AO/PI solution staining and observed under a light fluorescence microscope. (A) shows untreated *Acanthamoeba* sp. cells that remain viable and healthy, while in (B) shows *Acanthamoeba* sp. cells after being treated with hydrogen peroxide, H_2O_2, and in (E) that exposed to 5G frequency, both facing necrotic cell death and appearing in orange colouration and indicating non-viable cell. (C) and (D) shows *Acanthamoeba* sp. cell undergo autophagic cell death after exposure to 3G and 4G frequency, respectively (Magnification 400x).

Programmed cell death (PCD) is a genetically regulated process linked to consistent morphological and biochemical alterations, ranging from apoptosis to necrosis. Apoptosis occurs when there is a disruption in the physiology of mitochondria within cells, leading to changes in mitochondrial permeability and specific protease activators released from the mitochondria. Notably, the disturbance of the outer mitochondrial membrane leads to the translocation of cytochrome-C to the cytosol, subsequently bringing about the depolarization of the inner mitochondrial membrane [14].

Hence, observation of *Acanthamoeba* sp. cells under a fluorescence microscope was done involving acridine orange (AO) and propidium iodide (PI) to classify the mode of cell death that occurred. Untreated *Acanthamoeba* sp. cells showed green coloration by AO dye, indicating that cells are viable and healthy. However, cells subjected to non-ionizing radiation (NIR) frequency exhibited changes in both the internal organelles and the integrity of their membranes. Cells treated with 3G and 4G frequencies showed green coloration of cytoplasm and yellow-orange nucleus, as in Figure 3 (C) and (D), and supported that autophagic cell death occurred. Late autophagy occurs, leading to cell death that enables PI dyes to stain the nucleus.

AO dyes that are intact to nucleic acids and become green in colour when inserted into the double-strand of DNA in the nucleus of a cell. Moreover, the presence of PI dyes was detected by the orange coloration as it enters the cell through damaged plasma membrane, indicating that the cell membrane was broken. Healthy cells remain green r as PI dyes are not allowed to enter the cell as it is healthy and the plasma membrane is still in good condition. Cells are categorized as damaged cells when they show yellow coloration, indicates that early apoptosis has happened. All these different colourations were by reason of different content in pH of nuclei [15].

In this experiment, *Acanthamoeba* sp. cells were treated with different strengths of NIR frequency in a 6-well plate for 24 hours on different days to determine the mode of cell death using AO/PI staining. Figure 3 showed results obtained from AO/PI staining, where (A)

untreated cells appeared in green coloration, indicating that the membrane cell is still intact and viable. Figure 3 (B) and (E) showed orange colouration due to necrotic cell death occurring after being treated and exposed to H_2O_2 and 5G frequency, respectively. Necrosis cell death, causes complete damage to cells compared to autophagy and apoptosis. PI dyes penetrate the cell membrane of non-viable cells and bind to DNA in the nucleus had causing an orange colour to appear [16]. Hence, using this double staining AO/PI dye determines to mode of cell death that occurred in *Acanthamoeba* sp. upon exposure to NIR, based on the coloration shown.

DNA Laddering Technique

The determination mode of cell death in *Acanthamoeba* sp. cells upon exposure to different NIR strengths was determined by using gel electrophoresis at the DNA level of *Acanthamoeba* sp. cells. DNA laddering technique was done and clearly indicated the effect upon exposure to NIR with different frequencies, 3G, 4G, and 5G, towards the DNA structure of *Acanthamoeba* sp. cell. Figure 3 shows the 'ladder-pattern' obtained when subjected to an electrophoresis system after exposure, and DNA extraction was done.

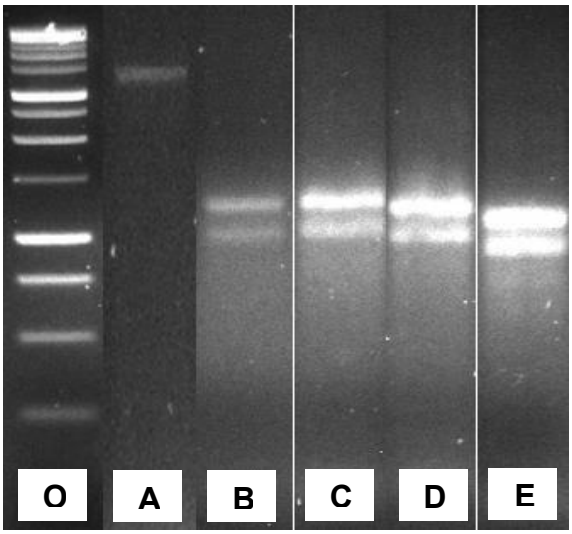

Figure 4. DNA ladders indicate the presence of DNA fragments that were viewed on an agarose gel. (O) indicate as the marker for the DNA laddering assay. DNA fragmentation in *Acanthamoeba* sp. cells in (A) shows that no DNA fragmentation occurs in untreated cells. In (B), (C), (D), and (E) clearly show that DNA fragmentation occurred upon treatment with hydrogen peroxide, H_2O_2, and exposed to different NIR frequencies 3G, 4G and 5G, respectively. Whereas the smeary background indicates random degradation of DNA fragments. The gel is the representative of at least three separate repeat experiments.

At a molecular level, stimulation of an endogenous nucleus induced DNA fragmentation, which produced a few pieces of varying lengths. Radiation exposure caused DNA double-strands to break into numerous pieces during the apoptosis process of cell death. Nonetheless, late random fragmentation displays a smeary backdrop formed with the release of lysosomal DNases, indicating necrotic cell death [17].
From this research, rapid DNA fragmentation was observed in untreated *Acanthamoeba* sp. cells and cells that were exposed to the NIR frequency. Based on the result obtained in Figure 4(A), no 'ladder-pattern' of DNA was obtained, and the DNA extraction stays at the top because of the heavy weight. Figure 4 (B), (C), (D), and (E) showed fragments of the target did underwent lysis, becoming lower molecular weight pieces, displaying the 'ladder-pattern' on agarose-gels. The smear and blurred background indicate that DNA fragmentation occurs outrageously upon being exposed to different strengths of NIR frequency. The image obtained from this technique supported that *Acanthamoeba* sp. undergoes autophagy and necrosis mode of cell death.

Conclusions

The result from the study on DNA integrity and mitochondrial function of *Acanthamoeba* sp. cells upon exposure to different non-ionizing radiation (NIR) frequencies – 3G, 4G, and 5G, showed that the radiation did affect the cells' morphological changes and cell's viability. Based on MTT assay results, it showed that the percentage of *Acanthamoeba* sp. cells decreased upon exposure to high NIR frequencies. 5G frequency obtained the lowest percentage of cell viability. The morphological changes of *Acanthamoeba* sp. cells can be seen under a light inverted microscope, and they differ from untreated cells. It showed that cells change from the trophozoite form into the cyst form after exposure. Autophagic and necrotic modes of cell death occurred after exposure to NIR and were observed using AO/PI staining under a light fluorescence microscope and filtered with a blue laser. DNA fragmentation from the gel electrophoresis showed that the DNA of cells undergoes lysis and has a low molecular weight to form a 'ladder-pattern'. This study only focused on morphological changes and determined the mode of cell death that occurs in *Acanthamoeba* sp. cells after exposure to different strengths of NIR frequency. Further confirmation in comparison of the number of cells before and after exposure to NIR by using MTT assay is highly recommended for a better final data to prove that the radiation causes a decrease in the number of cells. Thus, proving that the increasing strength of NIR caused severe effects on the *Acanthamoeba* sp. cell.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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