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Non-Thermal Biological Effect of Graphene Far-Infrared Ray on *Saccharomyces cerevisiae* Cells

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Abstract

Graphene materials can emit far-infrared ray, but the biological effects of graphene far-infrared ray have not been studied. Furthermore, the non-thermal biological effect of far-infrared ray on organism has not been systematically studied independently of the thermal effect. The purpose of this study was to investigate the non-thermal biological effect of graphene far-infrared ray (gFIR) on *Saccharomyces cerevisiae* cells. In this work, stringent control of the cultivation conditions was carried out to ensure the stability and constancy of the culture and its temperature. Flow cytometry was used to detect the non-thermal effect of gFIR irradiation on cell membrane permeability, mitochondrial membrane potential (MMP) and intracellular reactive oxygen species (ROS) content. Compared with the control group, cell membrane permeability of the gFIR exposure cells decreased by 4.7%, MMP increased by 16% and intracellular ROS reduced by 10.7%. The results revealed the valuable features of the non-thermal biological effect of gFIR on *Saccharomyces cerevisiae* cells, and the further analysis demonstrated that graphene far-infrared materials should have great application value in disease prevention and health promotion.

Keywords

Graphene Far-Infrared Ray, Non-Thermal Biological Effect, *Saccharomyces cerevisiae*, Cell Membrane Permeability, Mitochondrial Membrane Potential, Reactive Oxygen Species

1. Introduction

Far-infrared ray (FIR) is non-ionizing radiation which exerts beneficial biologi-

cal effects, including the increase of blood flow volume [1] [2], promoting the growth of skeletal muscle cells [3], increasing anti-oxidative stress capability [4], promoting wound healing [5] [6], diabetes treatment [7] [8], tumor suppression [9] [10], sleep modulation [11], neurodegenerative diseases treatment [12] [13] [14], cardiovascular disease treatment [15], etc. Most FIR radiation devices were accompanied by heat, but a large number of previous studies did not distinguish between the thermal and non-thermal effects of FIR. In research [16], the heat of FIR was removed to study the biological effects of FIR on VEGF-induced proliferation in human umbilical vein endothelial cells (HUVECs). To evaluate the thermal effect on cell proliferation, they cultivated VEGF-pretreated HUVECs at 38˚C - 39˚C without FIR exposure. They found that FIR exposure inhibited VEGF-induced proliferation in HUVECs, while pretreatment at 38˚C - 39˚C without FIR exposure slightly increased VEGF-induced proliferation in HUVECs. These results showed that the biological effects of FIR in HUVECs resulted from non-thermal effect of FIR. They further found that the low-temperature FIR irradiation inhibited advanced glycation end products (AGEs)-induced endothelial injury both in vivo and in vitro [17]. It is obvious that the non-thermal biological effect of FIR has potential application value in both scientific research and healthcare, and it is of great significance to study the non-thermal biological effect of FIR.

The range of FIR electromagnetic waves is 3 to 1000 μm as recommended by the International Commission on Illumination. In a recent study [18], in order to study the biologically effective wavelength of FIR, the researchers measured the migration and respiration of epithelial cells using two narrowband FIR sources (2 - 5.25 and 8 - 10 μm). They found that an FIR of 8 - 10 μm significantly increased cell migration and the basal and maximum respiration of mitochondria. On the contrary, the 2 - 5.25 μm FIR not only inhibited cell migration, but also did not promote mitochondrial function of the epithelial cells. Therefore, it was concluded that the biologically effective wavelength of FIR was 8 - 10 μm. This was the first report on the difference in biological effects in the far-infrared radiation band, indicating that the wavelength distribution of far-infrared materials may affect the biological effects. Since different far-infrared materials have their own wavelength distribution, it is necessary to study the biological effects of different types of far-infrared materials in detail.

Graphene is an advanced biological material, discovered in 2004 by physicists Konstantin Novoselov and Andre Geim of university of Manchester in the United Kingdom [19]. When heated to high temperatures, graphene emitted and absorbed FIR [20]. Based on this characteristic, various graphene far-infrared products have been developed, which are easily accessed online and offline. Graphene has a unique far-infrared band distribution, that is different from other far-infrared materials and leads to its distinctive biological effects. However, so far, there have been no reports on the non-thermal biological effect of gFIR.

Saccharomyces cerevisiae (*S. cerevisiae*) is characterized by rapid growth and easy cultivation. Nearly 31% of its protein coding genes are similar to mamma-

lian proteins. As a eukaryote, *S. cerevisiae* shares the complex internal cell structure of animals and human beings, and many important proteins of human were first discovered by studying their homologs in yeast. As one of the most intensively studied eukaryotic model organisms, for more than 5 decades, *S. cerevisiae* has been studied to better understand aging, senescence, stress response, radiation damage and so on.

In this study, we used *S. cerevisiae* cells to investigate the non-thermal biological effect of gFIR. We found that the non-thermal effect of gFIR decreased the cell membrane permeability, increased the mitochondrial membrane potential (MMP), and reduced the intracellular reactive oxygen species (ROS) content. We concluded that gFIR can affect *S. cerevisiae* cells through its non-thermal biological effect.

2. Materials and Methods

2.1. Cell Culture

The industrial instant dry yeast *S. cerevisiae* (Angel Yeast CO. Ltd., China), preserved in 15% glycerol at −70˚C as described by Sherman [21] was used in this study. Yeast cells were grown to logarithmic phase in YPD medium (1% yeast extract, 2% tryptone, and 2% D-(+)-glucose) for each experiment. All reagents were purchased from Sigma-Aldrich (Steinheim, Germany). All glassware and medium were autoclaved at 121˚C for 20 min.

Logarithmic grown cells were harvested by centrifugation; suspended in fresh YPD medium to obtain a cell culture of 2×10^7 cells/mL, and allocated evenly to 10 Erlenmeyer flasks of 50 ml, each with 20 ml cell culture. Five flasks with cell culture used as the exposure group were directly exposed to gFIR, while the other five flasks with cell culture used as the control group were shielded with aluminum sheets to avoid gFIR exposure. The room temperature was maintained at 26˚C ± 0.5˚C. All cell cultures were under strict and continuous monitoring to ensure the temperature was within the designed range. After 3 h irradiation, cell samples were withdrawn to detect cell membrane permeability, MMP and intracellular ROS content.

2.2. FIR Source

The graphene film from ENN Graphene Technology Co. LTD. was used in this study. This film was made up of 5-layer graphene with 0.338 mm in thickness. Activated and powered by standard 220 V AC, the film was proven to emit a waveband of 5 - 20 μm far-infrared ray with a peak wavelength of 6.5 - 9.5 μm. In order to investigate the non-thermal biological effect of gFIR, the glass flasks containing yeast cell culture were kept at a sufficient distance from the film to ensure the exposure group was at the same temperature as the control group.

2.3. Stain by Propidium Iodide (PI)

After 3 h of gFIR exposure, cells were collected in a microcentrifuge tube with a final concentration of 10^6 cells /mL. Cells were incubated with 2 μg/mL PI at

30˚C for 20 min in the dark, and then washed twice with PBS. Fluorescence was observed by Flow Cytometry (CytoFLEX, Beckman, China) with excitation and emission wavelengths of 488 and 564 - 606 nm, respectively (PE channel). For each sample, 10,000 events were recorded and data were reported as mean fluorescence intensity.

2.4. Detection of MMP

The changes in MMP were determined with Rhodamine 123 (Rho123), a cell permeable cationic dye. Rho123 dye was added to the cell suspension at a final concentration of 2 μg/mL, and the cells were further incubated at 30˚C for 20 min. Then the cells were washed twice with PBS and resuspended in PBS. The fluorescence was detected by Flow Cytometry (FITC channel).

2.5. Detection of Intracellular ROS

2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) (Beyotime, Nantong, China) fluorescent probe, which can freely enter into cells and interact with intracellular ROS to produce a highly fluorescent 2',7'-Dichlorodihydrofluorescin (DCF), was used to detect ROS in cells. After the cells were harvested, a certain amount of cells was washed twice with PBS. And then, DCFH-DA was added to the suspension liquid at a final concentration of 20 μM, incubated at 30˚C for 30 min. The fluorescence was detected by Flow Cytometry at excitation wavelength of 488 nm and emission wavelength of 505 - 545 nm (FITC channel).

2.6. Statistical Analyses

Data were presented as the mean ± SD. The difference between two groups was determined using student's paired t-test, and the $P < 0.05$ was considered statistically significant. All statistical analyses were performed with MICROSOFT EXCEL (2016).

3. Results and Discussion

Far infrared ray has strong penetration and radiation capabilities, and has a significant resonance effect. It was found that FIR absorption spectrum was significantly affected by water cluster size and temperature [22]. When the FIR was absorbed by the human body, it caused the resonance of water molecules. Subsequently, the water molecules were activated and the binding force between water molecules was enhanced. Proteins and other biomacromolecules were then activated by the active water molecules, keeping the cells at its highest vibration level. The realization of this resonance absorption required the interaction between appropriate FIR and living organisms. Different FIR wavelengths produced a different or even an opposite biological effect [18]. Many studies focused on materials such as ceramic powder, fiber products and tourmaline [23], however, the biological effects of graphene far-infrared materials have not been reported. In this study, non-thermal effect of gFIR was found to affect *S. cerevi-*

siae cells in several ways. In order to study the non-thermal effect of gFIR on *S. cerevisiae cells*, we first compared the growths of cells between the exposure group and control groups, and no significant difference was found. Then, we further investigated the cell viability (CCK8 staining), cell respiration rate (OCR), ATP content, cell membrane permeability, MMP and intracellular ROS content. Our data showed that gFIR affected cell membrane permeability, MMP and intracellular ROS content.

Natural apoptosis occurs during cell growth [24]. The increase of cell plasma membrane permeability is one of the cell biology characteristics of the natural apoptotic program. In this process, the selective permeability of the cell membrane decreases, the transport protein on the membrane loses its function, and the cells gradually lose their selective transport ability. When the integrity of the cell membrane is in healthy conditions, PI cannot pass through the cell membrane, however, if the integrity is destroyed, PI passes through the cell membrane and accumulate in the cytoplasm, emitting strong fluorescence. To evaluate the affection of gFIR on the exposure yeast cells, we investigated the cell membrane permeability by PI staining. Results showed that the fluorescence intensity of the exposure group decreased by 4.7% compared with the control group (**Figure 1**), implying that the non-thermal effect of gFIR could help to maintain the integrity of cell membranes and reduce the natural apoptosis in *S. cerevisiae* cells.

Mitochondria is the major energy source of cells, MMP is a key indicator of mitochondrial viability, so in this work, we detected MMP to study the effects of gFIR on mitochondria. The fluorescent probe Rho123 was used to detect MMP. The detection method relies on the fact that Rho 123 accumulates in membranes in a manner which is dependent on membrane polarization. Low MMP leads to the enhancement of dye penetration and the increase of fluorescence intensity. In contrast, in cells with high MMP levels, the penetrability of Rho123 was reduced, resulting in a reduction in fluorescence intensity. Fluorescence intensity of the gFIR exposure cells decreased by 16% compared with that of the control group (**Figure 2**), indicating that the exposure cells had high MMP. It was reported that high MMP can keep cells high viability, while low MMP or loss of MMP was a hallmark of apoptosis [25]. In the MMP evaluation experiment, the MMP of exposure group was significantly higher than that of control group, which illustrated that gFIR exposure was beneficial to sustain the cell MMP and keep the cells in a more active state.

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (·OH), are the most abundant free radicals in cells. They are formed as necessary by-product during aerobic respiration in normal intracellular metabolism, and play essential roles in physiological function in cells. When intracellular ROS production is within a normal controllable range, it acts as a signal transduction molecule, participating in cell differentiation, proliferation and host defense response. However, excessive production of intracellular ROS can damage cellular macromolecules such as lipids, proteins

and DNA, resulting in oxidative stress. It is well known that the excessive production of intracellular ROS directly related to many common diseases, including aging diseases [26], malignant tumors, cardiovascular diseases, diabetic complications and other inflammatory disorders [27]. Reducing intracellular ROS levels is an effective way to treat and prevent diseases induced by oxidative stress. Our experiments showed that the fluorescence intensity of the exposure group was reduced by 10.7% compared with the control group (**Figure 3**), indicating that the non-thermal effect of gFIR reduced the accumulation of ROS at the cellular level and improved the antioxidant capacity of yeast cells. From the perspective of intracellular ROS production and maintenance, it was also evident that gFIR exposure produced a very beneficial non-thermal biological effect on *S. cerevisiae* and even human beings.

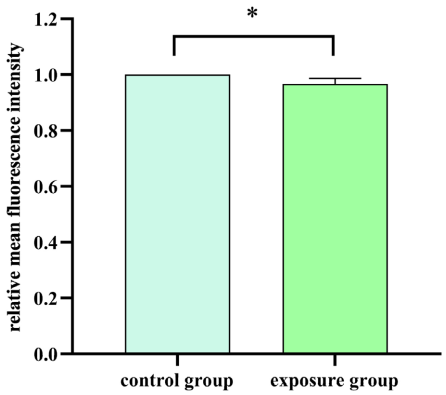

Figure 1. PI staining to evaluate the membrane permeability of *S. cerevisiae* cells in gFIR exposure group and control group. Mean fluorescence intensity was normalized, *i.e.*, in each experiment, the control group was defined as 1, and the fluorescence ratio between the exposure group and the control group was defined as the exposure group value. T-test was conducted on the relative mean fluorescence intensity of three independent experiments. *P < 0.05 indicated statistically significant differences between the exposure and the control group.

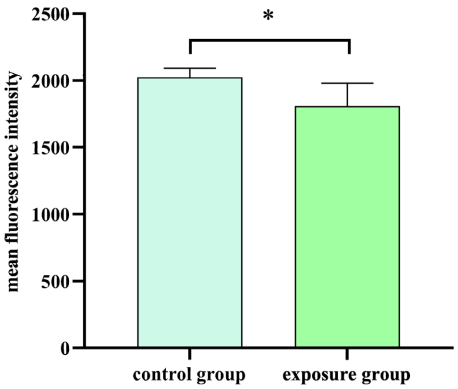

Figure 2. Rho123 staining to evaluate the MMP of *S. cerevisiae* cells in gFIR exposure group and unexposed control group. *P < 0.05 indicated statistically significant differences between the exposure group and the control group. Similar results were obtained in three independent experiments.

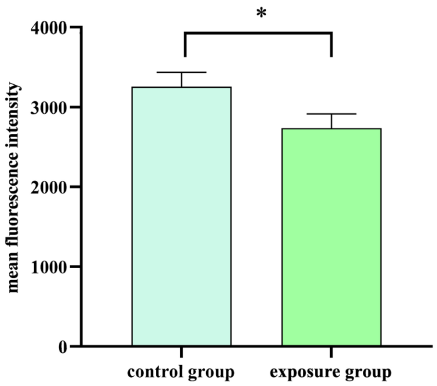

Figure 3. Measurement of the ROS of *S. cerevisiae* cells in gFIR exposure group and unexposed control group. *P < 0.05 indicated statistically significant differences between the exposure group and the control group. Similar results were obtained in three independent experiments.

4. Conclusion

In conclusion, the non-thermal effect of gFIR was beneficial to maintain the membrane selective permeability and MMP, reduce the accumulation of intracellular ROS, and eventually inhibit the natural apoptosis of cells. The possible mechanism was that gFIR caused the resonance of water molecules and macromolecules, subsequently affected oxidative respiratory chain and enhanced the ability of endogenous antioxidant enzymes, consequently decreasing the intracellular ROS level. Low level of intracellular ROS reduced the attack on the membrane system, maintained the permeability of the membrane, improved MMP and inhibited the natural apoptosis of cells. Although the exact mechanism remains to be studied, there is no doubt that the non-thermal effect of gFIR improves the state of cells in multi-aspect, and gFIR products have a positive impact on health maintenance. The results of this study came from gFIR materials, but we considered that this conclusion was also applicable to other types of far-infrared materials. In next step, oxidative respiratory chain and endogenous antioxidant enzymes would be studied to further understand the proposed mechanism behind the gFIR non-thermal biological effect.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

This article does not contain any studies involving animals or human partici-

pants performed by any of the authors.

Authors' Contributions

Miao Tian: Methodology, Formal analysis, Writing-Original draft. Qing Li: Methodology, Formal analysis, Writing-Review. Yong-dong Yang: Resources. Bruce tang: Project administration. Jin-lai Li: Conceptualization, Resources. Hong Wu: Conceptualization, Validation, Supervision, Writing-Review & Editing. All authors read and approved the final manuscript.

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