

Direct and Indirect Effects of Ceramic Far Infrared Radiation on the Hydrogen Peroxide-scavenging Capacity and on Murine Macrophages under Oxidative Stress

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Abstract

Far infrared (FIR) rays are used for many therapeutic purposes, but the intracellular mechanisms of their beneficial effects have not been entirely elucidated. The purposes of this study were thus to explore the effects of ceramic-generated far infrared ray (cFIR) on RAW 264.7 cells by determining the scavenging activity of hydrogen peroxide (H₂O₂), cell viability, and changes in cytochrome c levels and the NADP⁺/NADPH ratios. The results showed that the H₂O₂-scavenging activity directly increased by 10.26% after FIR application. Additional FIR treatment resulted in increased viability of murine macrophages with different concentrations of H₂O₂. cFIR significantly inhibited intracellular peroxide levels and LPS-induced peroxide production by macrophages. The increased ratio of hypodiploid cells elicited by H₂O₂ was significantly reduced by cFIR. The effects of cFIR on H₂O₂ toxicity were determined by measuring intracellular changes in cytochrome c levels and the ratio of NADP⁺/NADPH, and results showed that cFIR may block ROS-mediated cytotoxicity. In conclusion, data from this study suggest that cFIR may possess antiapoptotic effects by reducing ROS production by macrophages. We also review past articles related to the effects of oxidative stress from metabolically produced H₂O₂, and discuss possible beneficial effects of cFIR on living tissues.

Keywords: Ceramic-generated far infrared ray (cFIR), Hydrogen peroxide (H₂O₂) scavenging, Oxidative stress, Murine macrophages, Cytochrome c, Hypodiploid cell

1. Introduction

Far infrared (FIR) rays are electromagnetic waves with wavelengths within the infrared spectrum. FIR, especially at 8~14 μm, have many biological effects, including accelerated wound healing via fibroblast proliferation, enhanced immunity via leukocyte strengthening, and promotion of sleep [1-3]. Specific discoveries include a report by Shimokawa et al. [4] which showed that FIR-treated water increased the number of free tetrahedral water molecules or smaller-sized clusters. FIR absorption causes the collapse of water clusters, and the energy transfer may be converted into molecular vibrations. Also, by

measuring the scavenging activity against hydrogen peroxide (H₂O₂), Jeon et al. [5] showed that the antioxidant effects of rice hull extract are enhanced by thermal FIR irradiation. The benefits of FIR were proven in their work by an experimental model of H₂O₂-induced DNA damage in human lymphocytes, in which the rice hull extract decreased DNA strand breakage.

H₂O₂ is a byproduct of normal oxygen metabolism in the aerobic cells of animals and plants. All organisms possess peroxidases or enzymes to break down low concentrations of H₂O₂ into water and oxygen. However, the continuous production of H₂O₂ contributes to increased concentration of reactive oxygen species (ROS) within both the mitochondrial matrix and cytosol. The rate of H₂O₂ production in mitochondria is approximately 2% of the total oxygen uptake under physiological conditions [7].

This potentially damages mitochondrial components and initiates degradation. Therefore, the continuous generation of H₂O₂ during aerobic metabolism is harmful, and acts as a

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burden on living systems [6]. Superoxide and H_2O_2 are the major primary sources of ROS, and play a role in certain pathological processes, including neurodegeneration, aging, and heart and lung toxicity. Superoxide anion is mainly formed through one-electron reduction of O_2 of the respiratory chain, which is then catalyzed by superoxide dismutase to form H_2O_2 .

A variety of inflammatory stimuli, such as lipopolysaccharide (LPS), are thought to cause human diseases through elevating ROS production. Such observations support the notion that ROS play a critical role in causing certain human diseases, and thus agents with the ability to block ROS production have therapeutic potential and merit further development. The changes in the intracellular ratio of $NADP^+/NADPH$ reflect the adjustment of intracellular chemical reduction, and thus are related to ROS-mediated cytotoxicity (8).

On the other hand, the large amount of cytochrome c in the mitochondrial intermembrane space should oxidize all the superoxide produced by the respiratory chain and convert it back to oxygen [9]. In the past, reduction of cytochrome c by superoxide was employed to measure superoxide generation and oxidative stress [10].

In this study we investigated the effects of cFIR on intracellular H_2O_2 , cytochrome c, and the $NADP^+/NADPH$ ratio, which are indices of the presence of antioxidants [9-12].

2. Materials and methods

2.1 FIR ceramic powder

The FIR-emitting ceramic powder was micro-sized particles made from numerous ingredients consisting of mineral oxides and mineral salts, such as aluminum oxide, which was put together by the biomaterials laboratory of Taipei Medical University, Taiwan. An scanning electron microscope (SEM) image of the FIR ceramic powder used in this study is shown in Fig. 1 FIR emissivity, the ratio of radiation energy irradiated from the sample to an ideal black body as described by Plank's Law, was determined by a CI SR5000 infrared spectroradiometer. The emissivity spectrum of the FIR ceramic powder was in the wavelength range of 6 and 14 μm (Fig. 2) [1,2].

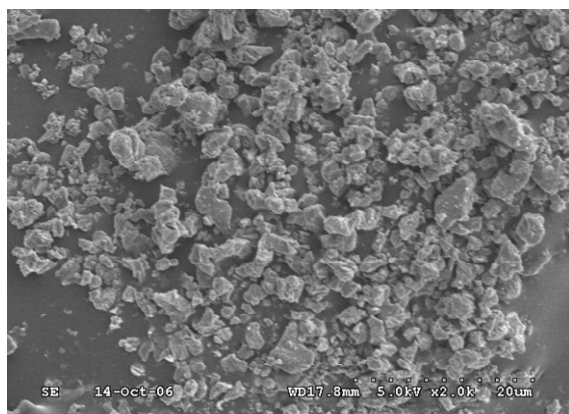


Figure 1. SEM picture of far-infrared ceramic powder used in this study.

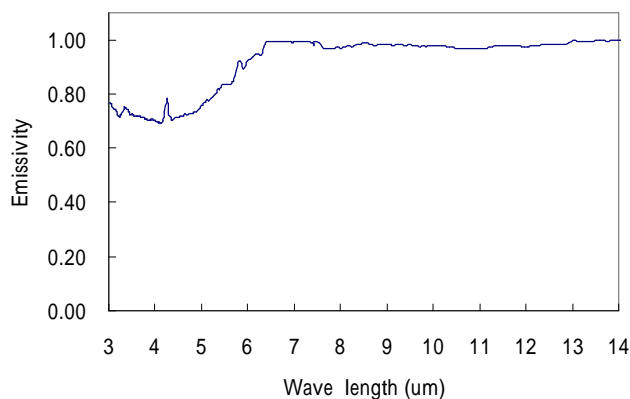


Figure 2. Emissivity spectrum of the far-infrared ceramic powder used in this study.

Equal amounts (100 g) of FIR-emitting ceramic powder were enclosed in different plastic bags (10 \times 20 cm) and used as the FIR irradiation source.

2.2 Direct scavenging of H_2O_2 with cFIR

An H_2O_2 solution (Sigma, St. Louis, MO, USA) with a concentration of 1 M was prepared, and equal 9 mL amounts of H_2O_2 solutions were added to the test tubes. The H_2O_2 solution was categorized into two groups: control and FIR. For the FIR group, test tubes were incubated at room temperature and covered externally by plastic bags filled with 100 g of the FIR ceramic powder for 3 h of irradiation. The control group was treated similarly, with the exception of FIR treatment. After the incubation period, the reagent (working concentration: 7.5 mM phenol red and 5 mg/mL horseradish peroxidase, Sigma, St. Louis, MD, USA) were added to each test tube. The mixture was allowed to react for 10 min, and the absorbance was observed at 550 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Gemini XPS Molecular Devices, Sunnyvale, CA, USA), with a lower absorbance value representing a higher H_2O_2 -scavenging ability.

2.3 Cell preparation

RAW264.7 cells, a mouse macrophage cell line, were obtained from the Bioresource Collection and Research Center (BCRC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin), and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Gaithersburg, MD, USA) and maintained in a 37°C humidified incubator containing 5% CO_2 . Cells were seeded until 80% confluent on the bottom of the dishes.

Equal amounts (100 g) of FIR-emitting ceramic powder or a control (non-functional powder) were enclosed in different plastic bag \times 20 cm) as the FIR irradiation source. Total cellular extracts were prepared according to our previous paper [1,2], separated on 8% \times 12% sodium dodecylsulfate (SDS)-polyacrylamide minigels, and transferred to immobilon polyvinylidene difluoride membranes (Millipore). Membranes were incubated with 1% bovine serum albumin (BSA) and then incubated with specific antibodies overnight at 4°C. The expression of protein was detected by staining with nitroblue

tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

In order to determine steady-state intracellular H_2O_2 levels, RAW264.7 cells were trypsinized and resuspended in ice-cold Hank's balanced salt solution (HBSS) containing 0.2 mg/mL of soybean trypsin inhibitor. Cell suspensions were preincubated for 5 min at 37°C and then treated with 100 mM DMNQ at 37°C. Aliquots were taken at different times, mixed with equal volumes of HBSS containing 10 mM DCFH-DA, and further incubated for 5 min at 37°C. The cellular fluorescence was then immediately determined by a flow cytometric analysis.

2.4 Determination of extracellular H_2O_2 production of RAW264.7 cells without LPS

RAW264.7 cells were incubated in a 5% CO_2 incubator for 24 and 48 h (1 and 2 days). After the cells had adhered to the dish, we replaced the medium, and then treated them with cFIR or control powder at 37°C in a 5% CO_2 incubator. We used an NWLSS™ Hydrogen Peroxide Assay kit (Northwest Life Science Specialties, LLC) to detect the extracellular H_2O_2 . The OD_{595} data represent the concentration of H_2O_2 within RAW264.7 cells.

2.5 Determination of extracellular H_2O_2 production induced by LPS in RAW264.7 cells

RAW264.7 cells were incubated in a 5% CO_2 incubator for 24 h. After the cells had adhered to the dish, we replaced the medium with one containing 1 µg/mL lipopolysaccharide (LPS; Sigma), then treated RAW264.7 cells with cFIR or control powder at 37°C in a 5% CO_2 incubator for 1 and 2 days. We then detected the extracellular H_2O_2 with the NWLSS™ Hydrogen Peroxide Assay kit. Data represent the OD_{595} within RAW264.7 cells.

2.6 Cell viability of cFIR-treated RAW264.7 cells under H_2O_2 -mediated oxidative stress

XTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 24 h, treated with different concentrations of H_2O_2 (100, 250, 500, and 1000 µM), and followed by a further 24 h of treatment. Cells were washed three times with phosphate-buffered saline (PBS; Gibco), and XTT (1 mg/mL) was added to the medium for 3 h, and the supernatant was then collected. The absorbance was read at 450 nm with an ELISA analyzer (Gemini XPS Molecular Devices, Sunnyvale, CA, USA).

2.7 Assay of hypodiploid cell analysis

RAW264.7 cells were seeded in six-well tissue culture plates at a density of 4×10^5 cells per well. After 24 h of culturing, the medium was changed and various concentrations of H_2O_2 (250 and 500 M) were added. For the FIR groups, the enclosed FIR ceramic powder was distributed uniformly in

plastic bags which were inserted beneath the tissue culture plates (Fig. 3).

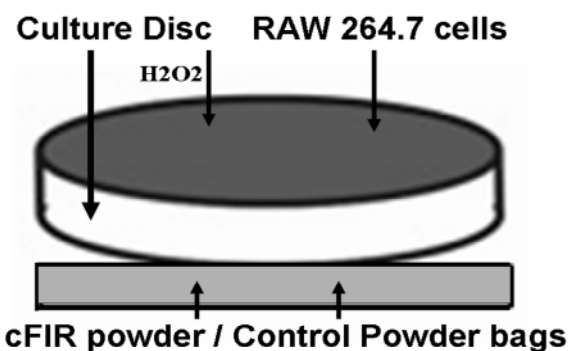


Figure 3. For cell culture, bags of ceramic-generated far-infrared powder and control powder were inserted beneath discs containing RAW264.7 cells to create FIR and control groups of cells.

Cells were treated with the indicated compounds for a further 24 h, and then were washed with PBS and stained with 3 µm propidium iodide (PI; Molecular Probes) for 30 min. The fluorescence emitted from the PI-DNA complex was quantitated after excitation of the fluorescent dye by FACSscan flow cytometry (Becton Dickinson).

2.8 Lactate dehydrogenase (LDH) activity release assay

The percentage of LDH activity release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in cells treated with lysis buffer (Roche). LDH release concentrations of the designated control and cFIR groups of H_2O_2 (400, 600, and 1000 µM)-treated cells were analyzed. After 6 h of incubation, the activity was monitored as the oxidation of NADH at 530 nm with an LDH assay kit (Roche).

2.9 Measurement of cytosolic cytochrome c levels

In order to identify cFIR's effect on cytosolic cytochrome c, RAW264.7 cells treated with FIR powder for 6 h and other untreated cells were then harvested by centrifugation at 3000 rpm for 5 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in five volumes of 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. Cells were homogenized and centrifuged at 1200 rpm for 10 min at 4°C to separate them into supernatant and pellets. The supernatant was then centrifuged at 12,000 rpm for 15 min at 4°C, and the obtained supernatant was used to identify of cytosolic cytochrome c by immunoblotting.

2.10 Measurement of $NADP^+$ / $NADPH$ levels

$NADP^+$ / $NADPH$ levels were measured by determining the rate of production of $NADP^+$ / $NADPH$, as previously described [13]. $NADP^+$ and $NADPH$ were assayed spectrophotometrically based on the measurement of the absorbance of the reduced coenzyme at 340 nm.

2.11 Statistical analysis

After H₂O₂ degradation, the cell viability and intracellular concentration of H₂O₂ were measured using a cell viability assay (XTT). We also performed an analysis of hypodiploid cells and an LDH release assay, and determined cytosolic cytochrome c and NADP⁺/NADPH levels. The statistical relationship between groups was determined using a *t*-test, with *p* values of < 0.05 considered significant.

3. Results

3.1 Direct scavenging of H₂O₂ with cFIR

Figure 4 shows that the mean absorbance of the control and FIR groups were 0.139 and 0.122, respectively (*n* = 25). The extent to which H₂O₂ disappeared in the FIR group was significantly larger than that of the control group, with a decrease of 12.23% (*p* < 0.001). This result confirms that H₂O₂ can be directly scavenged by FIR treatment.

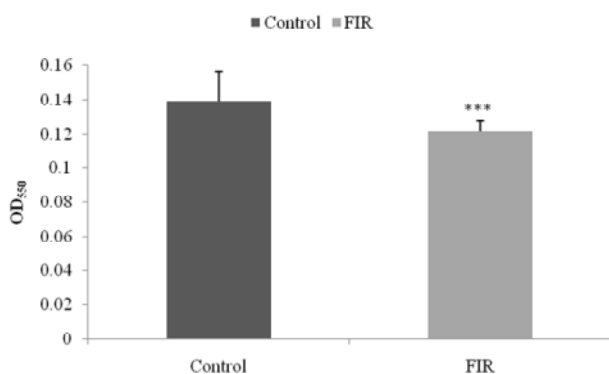


Figure 4. Comparison of H₂O₂ content in direct H₂O₂ scavenging with ceramic-generated far-infrared. ****p* < 0.001 indicates a significant difference compared with the control group.

3.2 Extracellular H₂O₂ production of RAW264.7 cells without LPS induction

As shown in Fig. 5, significant decreases in the peroxide level by cFIR were identified after both 1 and 2 days (*p* < 0.05).

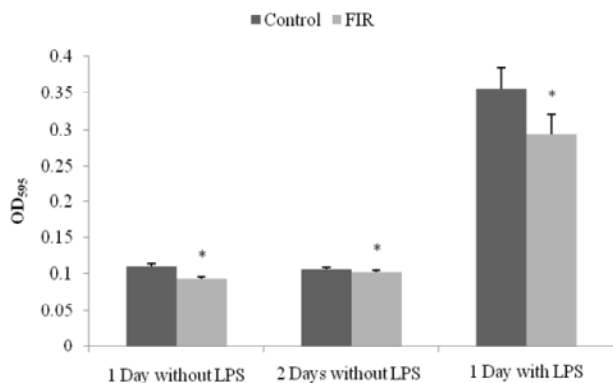


Figure 5. Significantly decreased in H₂O₂ by ceramic-generated far-infrared irradiation with and without lipopolysaccharide stimulation. **p* < 0.05 indicates a significant difference compared with the control group.

3.3 Extracellular H₂O₂ production of RAW264.7 cells with LPS induction

The peroxide level induced by LPS was significantly decreased by cFIR (*p* < 0.05) (Fig. 5).

3.4 Cell viability of cFIR-treated RAW264.7 cells under H₂O₂-mediated oxidative stress

Additionally, cFIR possessed the ability to stimulate the proliferation of RAW264.7 cells, according to the XTT assay (Fig. 6). The percentage of cell proliferation differed significantly when cells were treated with 250 μm H₂O₂ (*p* < 0.05).

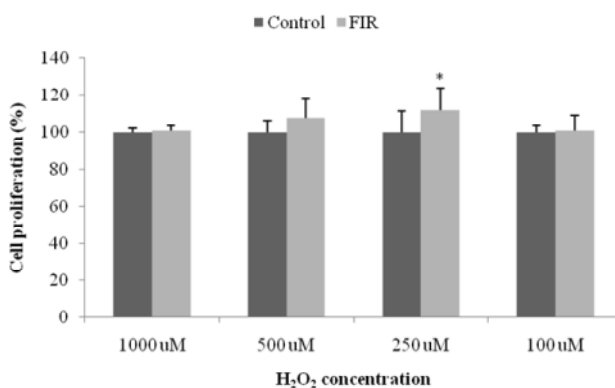


Figure 6. With different concentrations of H₂O₂ as the source of oxidative toxicity, cFIR possesses the ability to stimulate the proliferation of RAW264.7 cells against H₂O₂, according to the XTT assay. **p* < 0.05 indicates a significant difference compared with the control group.

3.5 Hypodiploid cell analysis and LDH activity release assay

The effect of cFIR on apoptosis induced by H₂O₂ was evaluated by flow cytometry with PI staining. As shown in Table 1, the ratio of hypodiploid cells increased in H₂O₂-treated cells, and this was significantly reduced by cFIR (*p* < 0.05).

The effect of cFIR on LDH release assays indicated a significant difference between the control and cFIR groups for H₂O₂-treated cells (400 and 600 μm, *p* < 0.05); the cFIR group showed a significant reduction in LDH release (Table 1).

Table 1. The effect of cFIR on the apoptosis induced by H₂O₂ was evaluated by flow cytometry via PI staining. The ratio of hypodiploid cells (percentage) is increased in H₂O₂-treated cells, and was significantly reduced by cFIR; and LDH release assay (cytotoxicity) indicated a significant difference between the groups, while it showed that a significant reduction in LDH release in the cFIR group for H₂O₂-treated cells.

	Percentage (%)		Cytotoxicity (%)		
	400 μm	600 μm	400 μm	600 μm	1000 μm
Control	30.5 ± 3.9	43.8 ± 4.7	135.2 ± 7.8	136.7 ± 9.8	131.9 ± 10.4
FIR	21.3 ± 2.5*	30.7 ± 3.8*	122.1 ± 7.3*	116.6 ± 3.7**	116.8 ± 10.4

p* < 0.05, *p* < 0.01 indicate a significant difference compared with the control group.

The cytoprotective effect of cFIR against ROS-induced cell death was predicted. The morphology of cells was observed under microscopy by demonstrating the apoptotic features of cells, including irregular cytoplasmic membranes

and chromatin condensation. These characteristic findings of hypodiploid cells after cFIR treatment were obviously lower than those in the control group (Fig. 7).

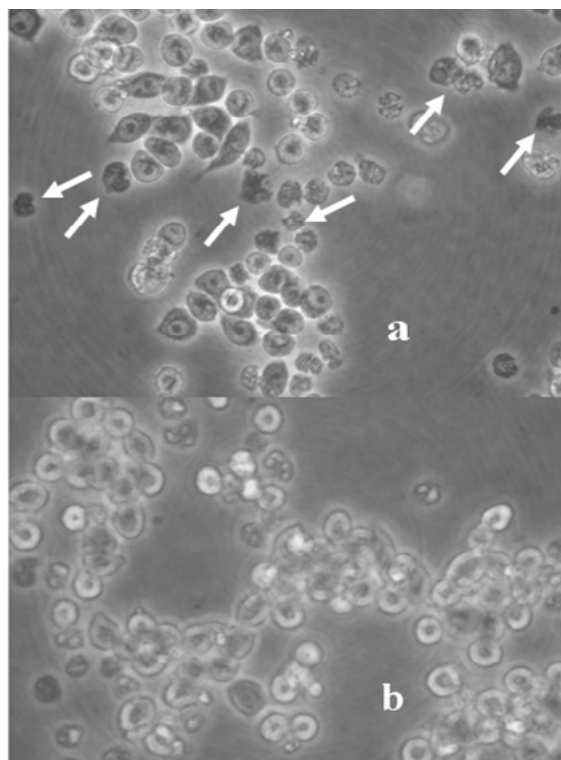


Figure 7. Effects of morphological changes induced by H_2O_2 on RAW264.7 cells by observing the morphology of cells under microscopy with a 200x power field. Cells were treated (a) without and (b) with cFIR in the presence of H_2O_2 , and hypodiploid cells are indicated with white arrows.

3.6 Cytosolic cytochrome *c* levels

ROS reduction by cFIR was investigated. As shown in Fig. 8, a significant decrease in H_2O_2 levels by cFIR was identified ($p < 0.05$). The results of a flow cytometric analysis showed that the intracellular peroxide level was reduced by cFIR using DCHF-DA as a peroxide-sensitive fluorescent dye.

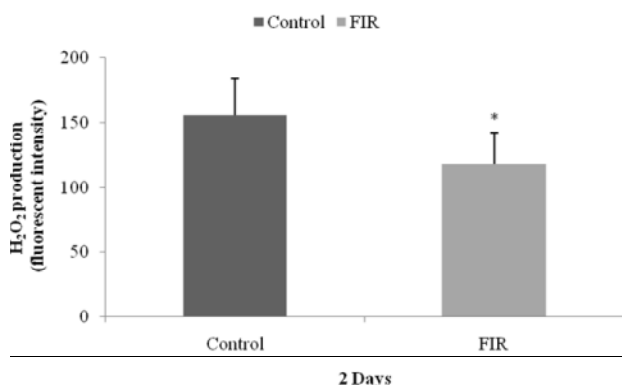


Figure 8. Flow cytometric analysis showing that levels of intracellular H_2O_2 were reduced by ceramic-generated far infrared via a flow cytometric analysis using DCHF-DA as a peroxide-sensitive fluorescent dye. * $p < 0.05$, indicates a significant difference compared with the control group.

The level of cytochrome *c* in the cFIR-irradiated groups of cells was found to have increased significantly via an immunoblotting analysis ($p < 0.01$; Fig. 9).

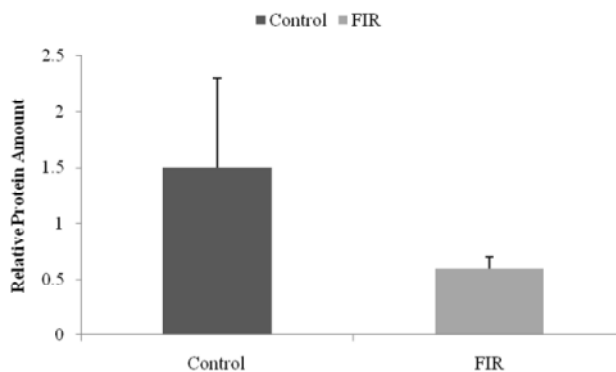


Figure 9. Immunoblotting analysis revealed that the level of cytochrome *c* in cFIR-irradiated groups of cells significantly decreased. ** $p < 0.01$ indicates a significant difference compared with the control group.

3.7 $NADP^+/NADPH$ levels

The $NADP^+/NADPH$ ratio was increased in the cFIR group (control group: 0.24 vs. cFIR group: 0.41). Through the effects of cFIR, more NADPH was consumed and was associated with an increase in $NADP^+$, which reflects an NADPH-reducing process.

4. Discussion

FIR can break hydrogen (H-O) bonds by exciting 'stretching' or 'bending' vibrations in water clusters [4], and thus decreases the size of water clusters. Hydrogen bonds are a factor that decreases the volatility of any liquid possessing them. This is related to the reduction rate of H_2O_2 , which exists in a cluster form in general conditions, rather than as single molecules. Therefore, the weakening of hydrogen bonds by FIR may also explain why FIR accelerates H_2O_2 transformation and releases H_2O and O_2 from H_2O_2 molecular clusters.

The H_2O_2 -scavenging capacity elicited by FIR-emitting ceramic materials may have beneficial biological effects. H_2O_2 is continually produced in oxidation-redox centers in both animal and plant aerobic respiratory systems. Cumulative increases in H_2O_2 and superoxide radicals can damage cells, including proteins, lipids, and DNA, leading to proven augmented mutation rates [14]. Reactive oxygen radicals of H_2O_2 are an important factor in oxidative stress, and are related to the pathogenesis of many important diseases in both animals and plants [15-16].

FIR is the major heat-transmitting radiation at wavelengths 3 μm to 1 mm, as defined by the CIE (1987). FIR, especially in the range of 3~14 μm , has many biological effects. Previous studies demonstrated that FIR has a wide range of applications, including increasing microcirculation, and its health-promoting properties are attracting more attention [1-2,17]. However, the mechanisms underlying these biological effects are still poorly understood. To our knowledge, there were no previous reports investigating the

scavenging ability of FIR and its antitoxic effects toward H_2O_2 . In our experiment, we proved that H_2O_2 can be scavenged directly by FIR treatment.

H_2O_2 is an oxygen species that causes the death of normal human fibroblasts exposed to an external source of oxygen radicals. Normal human cells may be damaged by phagocyte-released oxygen radicals at the site of inflammation and from sources other than phagocytic cells [18]. In pathophysiological conditions, H_2O_2 is continuously generated, and levels thus remain higher than normal [19]. Although speculative, the concentrations of H_2O_2 used in this study may be within the range that occur in some pathological states. H_2O_2 gradients form if the H_2O_2 production site and H_2O_2 degradation site are separated by membranes. Because H_2O_2 is highly membrane-permeable, the equilibrium between extra- and intracellular levels of H_2O_2 is reached extremely rapidly, or within 1 s, such that the measurement of intracellular levels of H_2O_2 also reflects extracellular levels [20]. Exposure of cells to H_2O_2 leads to cell death by apoptosis and necrosis. Cell death or cytotoxicity is classically evaluated by quantifying plasma membrane damage.

In our experiment, we also used LDH to prove the antioxidant ability of cFIR in circumstances of H_2O_2 toxicity. LDH is a stable enzyme which is present in all cell types, and it is rapidly released into the cell culture medium when the plasma membranes are damaged. LDH is thus the most widely used marker in cytotoxicity studies. Pathological or aging-related overproduction of mitochondrial ROS may lead to activation of apoptotic pathways [21]. Protection of cells from these intracellular oxygen radicals appears to be due to the presence of a variety of intracellular enzymes and naturally occurring radical scavengers. Under normal conditions, these protective mechanisms are adequate to prevent extensive damage to vital cellular constituents. Excessive ROS cause the degenerative diseases of aging, particularly cancer and atherosclerosis, as consequence of oxidative damage [22-24].

We selected RAW264.7 murine macrophages as our target cells because macrophages are vital for recognizing and eliminating microbial pathogens, and the survival of macrophages directly contributes to a host's defense system. Several previous studies showed that the virulence of some bacteria is due to their ability to trigger the death of activated macrophages by stimulating ROS production. Therefore, investigating protective mechanisms and developing agents with the ability to protect macrophages from ROS insults is an important issue [25]. RAW264.7 macrophages play a significant role in innate immunity and inflammation, and when activated by pathogens and cytokines they produce large amounts of H_2O_2 and ROS, exerting strong cytotoxicity against microorganisms and many cells, including killing macrophages themselves [26]. Therefore, increasing the survival rate of macrophages engaged in defensive processes against pathogens and cancer cells would enhance cell-mediated immunity [27].

Mitochondria are the main source of ROS in most aerobic mammalian cells, and cytochrome c in the respiratory chain is essential for maintaining a lower physiological H_2O_2 concentration in mitochondria [11]. Cytochrome c is thus an

ideal antioxidant that attacks superoxide and oxygen radicals. In living cells, the generation of oxygen radicals and H_2O_2 in the respiratory chain is a result of electron leakage, and the levels of oxygen radicals and H_2O_2 are kept in a balanced state between generation by the respiratory chain and elimination by cytochrome c. Therefore, a lack of cytochrome c within the respiratory chain causes higher levels of oxygen radicals and associated H_2O_2 accumulation [28-30]. Our results demonstrated that cFIR enhanced the antioxidant effect of H_2O_2 by consuming more intracellular cytochrome c.

On the other hand, calmodulin (Cam) increases the rate at which NADPH-derived electrons are transferred to enzyme flavins, and also triggers electron transfer and the oxidation of NADPH to $NADP^+$. Meanwhile, the activity of cytochrome c acting as a reductase promotes the activation of NO synthesis from L-arginine [31].

This is the first study exploring the possibility that cFIR may exhibit antioxidant characteristics in mammalian cells via its effects on intracellular levels of H_2O_2 , and the levels of cytochrome c and $NADP^+/NADPH$. We previously showed that cFIR induces intracellular levels of Cam and NO in RAW264.7 cells [2]. Summing up all of our data, we envision a possible pathway through which cFIR might exert its antioxidant effect (Figure 10). However, the in vitro experiments described in this work are not a perfect model to examine the influence of FIR-emitting ceramic materials on in vivo H_2O_2 production. In the future, a more-precise measurement method to detect tiny differences in the cellular H_2O_2 -scavenging capacity needs to be developed.

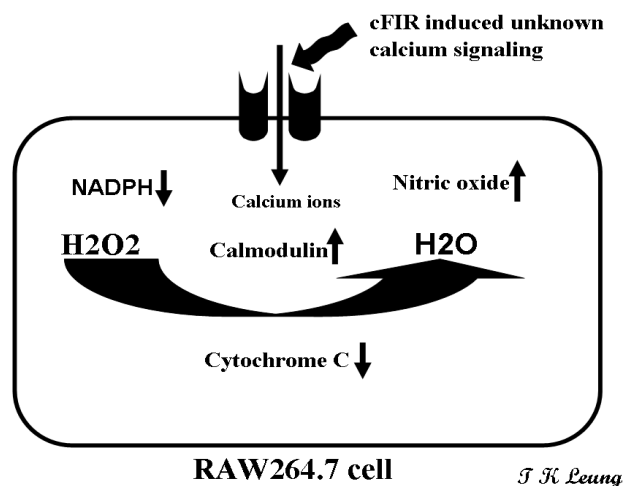


Figure 10. A possible pathway through which ceramic-generated far infrared might achieve its antioxidant effects.

5. Conclusions

The present study first explores how to use a physical method of cFIR irradiation that contributes to H_2O_2 -scavenging effects in cells. We correlated the results of cell viability, hypodiploid cell analysis, LDH release assay, and the cytochrome c and $NADP^+/NADPH$ levels of the RAW264.7 cell line (murine macrophages). Our results demonstrated that the group of cells exposed to cFIR significantly differed from the control group. It thus may be worth applying cFIR for its

antioxidant, anti-aging, immunity-boosting, and other related health-promoting effects. However, to better elucidate the precise effects of cFIR on living systems, it is important that future research develops an in vivo experimental model. We believe that these results justify further work to develop a more mature explanation of the biomolecular mechanisms of cFIR with regard to mammalian cells.

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