Photobiomodulation with 670 nm light increased phagocytosis in human retinal pigment epithelial cells

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Purpose: Photobiomodulation is the treatment with light in the far-red to near-infrared region of the spectrum and has been reported to have beneficial effects in various animal models of disease, including an age-related macular degeneration (AMD) mouse model. Previous reports have suggested that phagocytosis is reduced by age-related increased oxidative stress in AMD. Therefore, we investigated whether photobiomodulation improves phagocytosis caused by oxidative stress in the human retinal pigment epithelial (ARPE-19) cell line.

Methods: ARPE-19 cells and human primary retinal pigment epithelium (hRPE) cells were incubated and irradiated with near-infrared light (670 nm LED light, 2,500 lx, twice a day, 250 s/per time) for 4 d. Next, hydrogen peroxide (H_2O_2) and photoreceptor outer segments (POS) labeled using a pH-sensitive fluorescent dye were added to the cell culture, and phagocytosis was evaluated by measuring the fluorescence intensity. Furthermore, cell death was observed by double staining with Hoechst33342 and propidium iodide after photobiomodulation. CM-H_2DCFDA, JC-1 dye, and CCK-8 were added to the cell culture to investigate the reactive oxygen species (ROS) production, mitochondrial membrane potential, and cell viability, respectively. We also investigated the expression of phagocytosis-related proteins, such as focal adhesion kinase (FAK) and Mer tyrosine kinase (MerTK).

Results: Oxidative stress inhibited phagocytosis, and photobiomodulation increased the oxidative stress-induced hypoactivity of phagocytosis in ARPE-19 cells and hRPE cells. Furthermore, H_2O_2 and photobiomodulation did not affect cell death in this experimental condition. Photobiomodulation reduced ROS production but did not affect cell viability or mitochondrial membrane potential. The expression of phosphorylated MerTK increased, but phosphorylated FAK was not affected by photobiomodulation.

Conclusions: These findings indicate that near-infrared light photobiomodulation (670 nm) may be a noninvasive, inexpensive, and easy adjunctive therapy to help inhibit the development of ocular diseases induced by the activation of phagocytosis.

Age-related macular degeneration (AMD) is a progressive and degenerative eye disease that is a common cause of vision loss in developed countries [1]. AMD is classified into dry and wet types, and the main clinical feature common to both is the accumulation of lipofuscin in retinal pigment epithelium (RPE) cells. Wet AMD is characterized by abnormal angiogenesis, and dry AMD is characterized by atrophy of the outer retinal layers and RPE cells [2,3]. Risk factors for AMD, such as aging, light damage, smoking, genetic factors, and oxidative stress, have been reported to cause the accumulation of lipofuscin [4].

RPE cells help maintain the normal functions of photoreceptor cells by playing a role in phagocytosis, a part of the visual cycle, by forming a blood–retinal barrier that permits the exchange of waste products and nutrients between the blood and the retina [5,6]. Phagocytosis by RPE cells is an essential function of homeostasis in the retina. Photoreceptor cells are damaged by exposure to light. RPE cells can remove deteriorated photoreceptor outer segments (POS) via phagocytosis to preserve the function of photoreceptor cells [7]. It is important that the phagocytosis of RPE cells is not inhibited because the dysfunction of phagocytosis can trigger RPE damage and lysosomal disorder that prevents the breakdown of waste products, ultimately resulting in the accumulation of lipofuscin [8,9]. Thus, we believe the inhibition of lipofuscin accumulation is likely to improve AMD [10]. Phagocytosis of POS by RPE cells involves several steps, such as binding, uptake, and degradation, and each step is regulated by some proteins. Phagocytosis of POS by RPE cells requires αvβ5 integrin for binding [11]. Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase and is phosphorylated by integrin engagement. Finnemann et al. have shown that POS binding by RPE cells increases FAK complex formation with αvβ5 integrin and activates FAK [11,12]. FAK is related to the binding of POS, whereas Mer tyrosine kinase (MerTK) is not required for binding but for internalization [12,13]. Rat RPE cells expressing MerTK can bind to the surface of RPE cells but have no effect on the internalization of POS [14].

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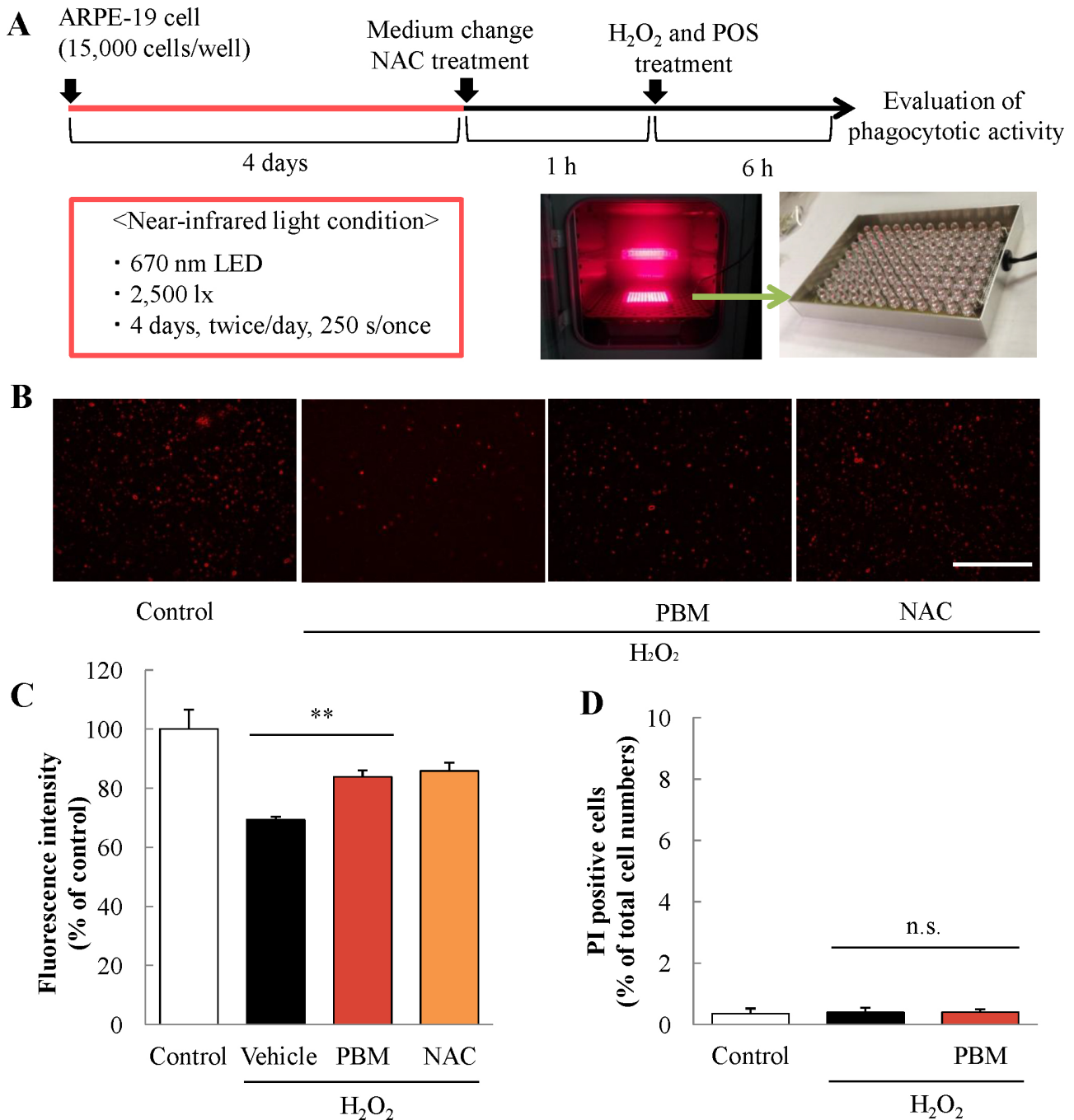
Figure 1. Photobiomodulation enhanced the phagocytic activity in oxidative stress. **A**: ARPE-19 cells were seeded at a density of 15,000 cells/ well and incubated for 4 day. During this period, they were irradiated with near-infrared light. After 4 day, the medium was changed and H_2O_2 and POS were added to the cells. Subsequently, images were collected using a fluorescence microscope. **B**: Oxidative stress reduced the phagocytic activity and photobiomodulation improved the phagocytic activity induced by oxidative stress. NAC used as positive controls also improved phagocytosis. Scale bar=500 μm. **C**: Quantification of fluorescence intensity was calculated as described in the Methods section. The data are expressed as means ± SEM (n=6) **p<0.01 versus the H_2O_2 only group. PBM: photobiomodulation; NAC: *N*-acetylcysteine.
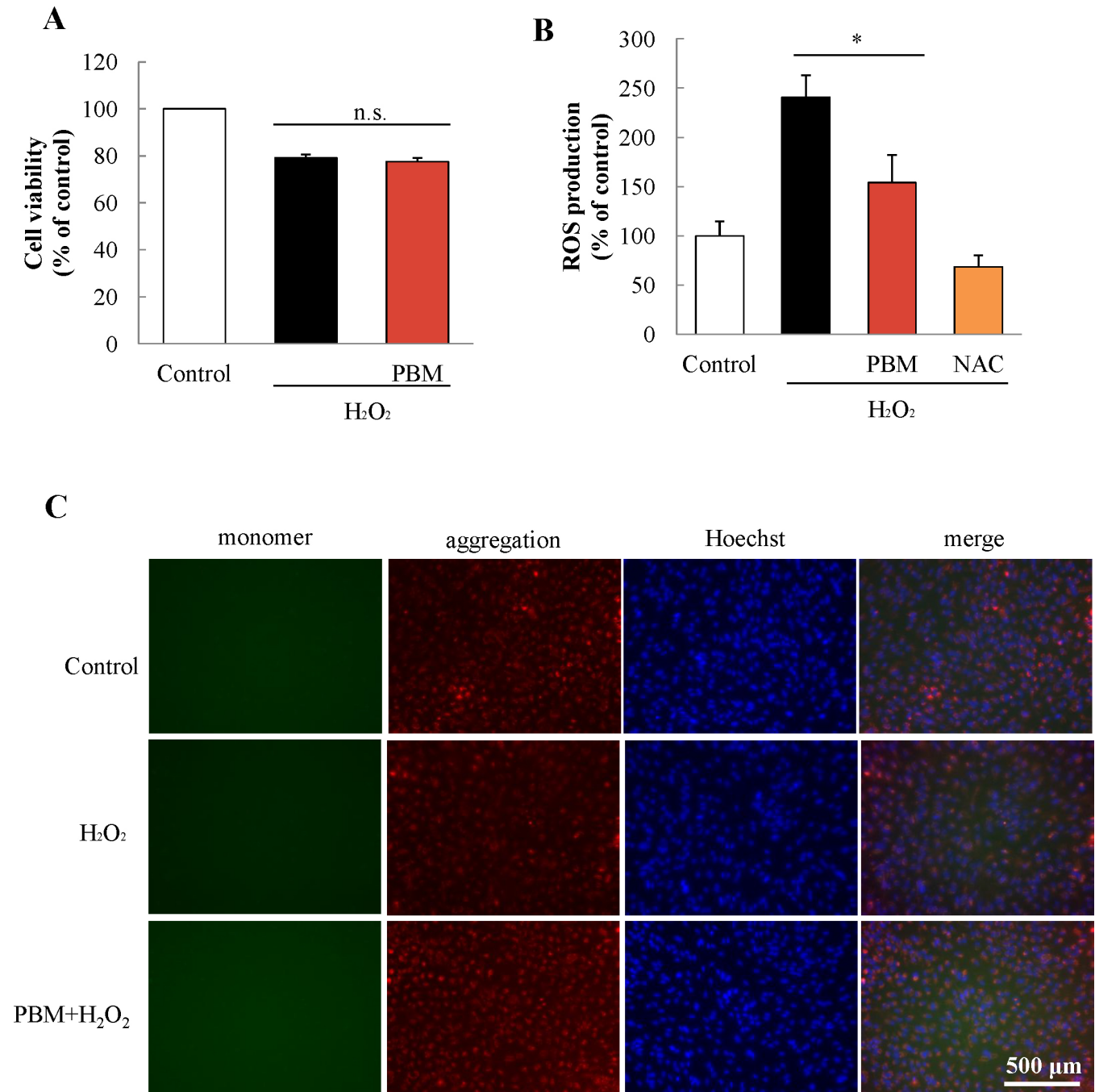
Figure 2. Photobiomodulation reduced ROS production but did not change the cell viability or mitochondrial membrane potential. **A**: Cell viability was measured using a WST-8 assay kit. Cell viability was reduced by oxidative stress, and photobiomodulation did not change the cell viability compared to the H_2O_2 only group. In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. **B**: ROS production was measured using the general oxidative stress indicator, CM-H_2DCFDA. Oxidative stress significantly increased ROS generation, and photobiomodulation reduced ROS production. NAC used as a positive controls also reduced ROS production compared to the H_2O_2 only group. In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. **C**: Mitochondrial membrane potential was evaluated using JC-1 dye. Green fluorescence indicates the low mitochondrial membrane potential, and red fluorescence indicates the low mitochondrial membrane potential. Oxidative stress and photobiomodulation did not change the mitochondrial membrane potential. Scale bar=500 μm. The data are expressed as means ± SEM (n=5 or 6) *p<0.05, **p<0.01 versus the H_2O_2 only group. In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. PBM: photobiomodulation; NAC: *N*-acetylcysteine.
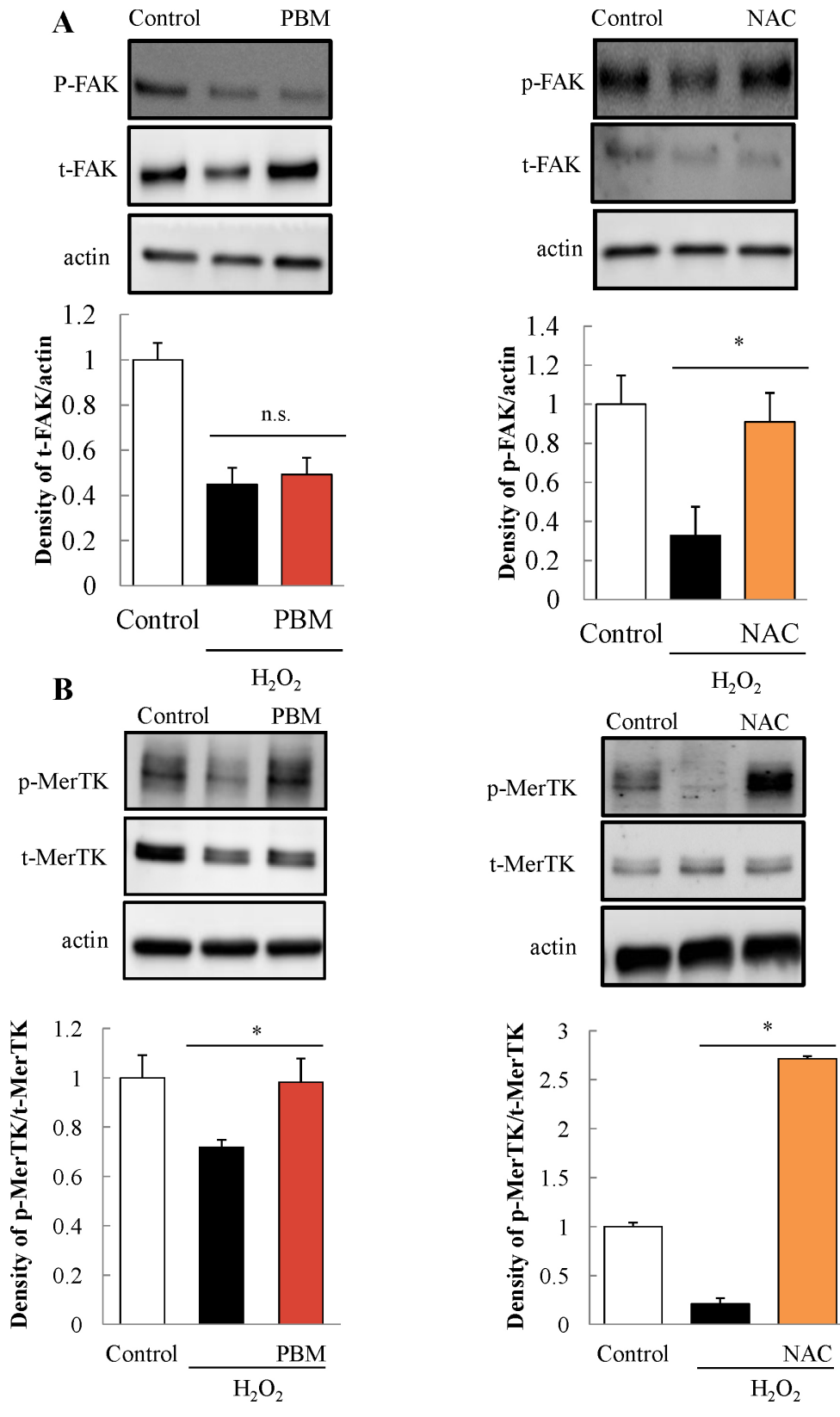
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Figure 3. Photobiomodulation increased the expression level of phosphorylated MerTK but not phosphorylated FAK. FAK and MerTK expression was measured by western blot. The quantification of the expression of phosphorylated FAK and phosphorylated MerTK was corrected by β-actin and total MerTK, respectively. **A**: Oxidative stress reduced phosphorylated FAK expression, and photobiomodulation did not change the expression of phosphorylated FAK. NAC increased the expression of phosphorylated FAK. **B**: Oxidative stress also reduced the expression of phosphorylated MerTK, and both photobiomodulation and NAC increased the oxidative stress-induced reduction of phosphorylated MerTK expression. The data are expressed as means ± SEM (n=4–10) *p<0.05 versus the H_2O_2 only group. In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. PBM: photobiomodulation; NAC: *N*-acetylcysteine.
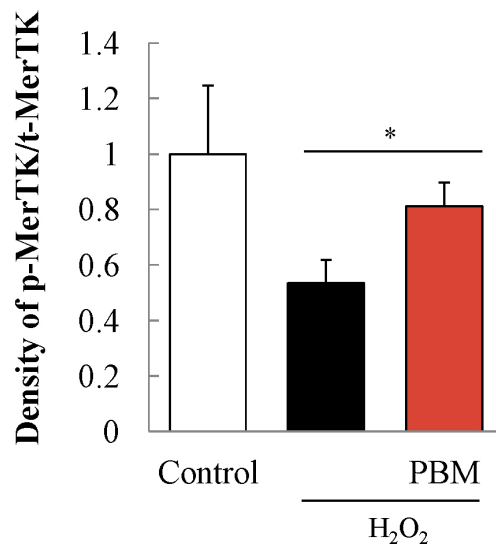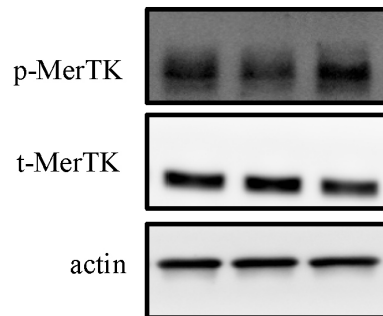
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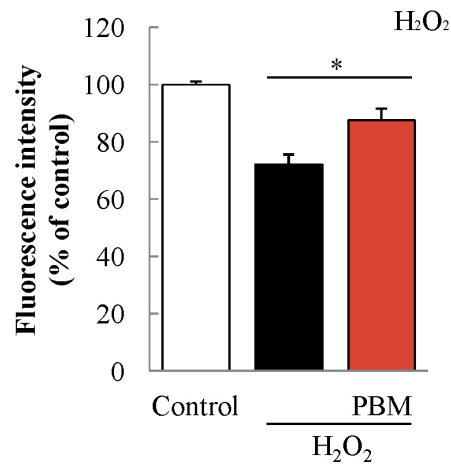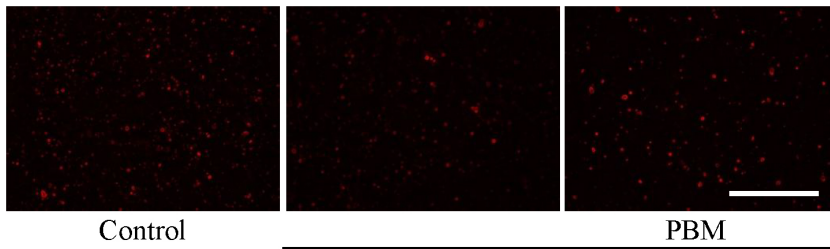
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Figure 4. Photobiomodulation enhanced the phagocytic activity in human retinal pigment epithelium cell cultures. **A**: Oxidative stress reduced the phagocytic activity, and photobiomodulation improved phagocytic activity induced by oxidative stress in hRPE cells. Scale bar=500 μm. **B**: Oxidative stress reduced the expression of phosphorylated MerTK, and photobiomodulation increased the oxidative stress-induced reduction of phosphorylated MerTK expression. The data are expressed as means ± SEM (n=6) **p<0.01 versus the H_2O_2 only group. In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. PBM: photobiomodulation.

The AMD models were appeared to mitochondrial dysfunction. The accumulation of lipofuscin decreased the mitochondrial membrane potential, impaired oxidative phosphorylation in the mitochondrial respiratory chain, and decreased the activity of phagocytosis [15].

Previous reports have shown that photobiomodulation, treatment with light in the far-red to near-infrared region of the spectrum, is beneficial in treating strokes, wounds, infection, diabetic retinopathy, and AMD [16-19]. Its beneficial effects include regulating cell viability by absorbing near infrared light in photosensitive molecules such as water, melanin, hemoglobin, and cytochrome *c* oxidase molecules. Some of the most important photosensitive molecules that respond to photobiomodulation are cytochrome *c* oxidase molecules, which accept electrons and are involved in producing adenosine triphosphate (ATP) in mitochondrial oxidative phosphorylation [20]. It is known that photobiomodulation activates cytochrome *c* oxidase and increases cellular ATP, resulting in the protection of neurons [21]. It has been reported that photobiomodulation suppresses the inflammation caused by decreased mitochondrial activity [16]. Other reports have suggested that photobiomodulation increases the expression of the antioxidant enzyme MnSOD without

affecting cytochrome *c* oxidase activity (which is related to mitochondrial activity); thus, the mechanism of photobiomodulation effects is not clear [17]. However, it is well known that photobiomodulation has effects such as the upregulation of ATP, the increase of antioxidant materials, and the prevention of inflammation in retinal neurons [17,22,23]. However, the effect of photobiomodulation on phagocytosis in RPE cells remains unclear. In the present study, we therefore investigated the effect of photobiomodulation on the oxidative stress-induced hypoactivity of phagocytes in ARPE-19 cells and primary human RPE (hRPE) cells.

METHODS

Cell culture: The human retinal pigment epithelial cell line (ARPE-19) was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM)/F-12 (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The ARPE-19 cells were passaged by trypsinization every 3–4 d.
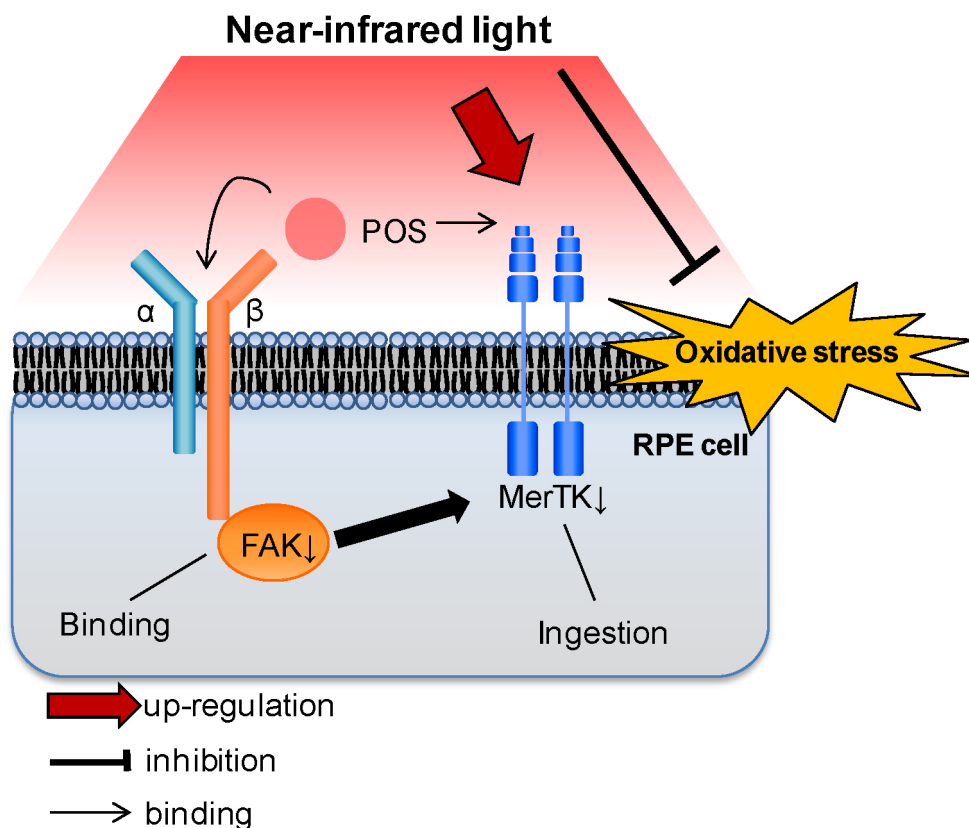

Figure 5. Photobiomodulation enhances phagocytosis via the upregulation of MerTK and has an anti-oxidative effect. Phagocytosis has several steps: binding to integrin, ingestion, and degradation of POS. FAK is thought to be involved in binding to integrin, and MerTK is thought to be related to the uptake of POS. Oxidative stress impairs the functions of RPE cells, such as phagocytosis. Our findings suggest that photobiomodulation can have an anti-oxidative effect and can activate MerTK. POS: photoreceptor outer segments; RPE: retinal pigment epithelium; FAK: focal adhesion kinase; MerTK: Mer tyrosine kinase (the big red arrow indicates upregulation and the normal arrow indicates "binding to receptors").

The primary hRPE cells were obtained from Lonza (Walkersville, MD). The cells were maintained in Retinal Pigment Epithelial Basal Medium (Lonza) containing 2% FBS, 4 mL L-glutamine (Lonza), 0.2 ml GA-1000 (Lonza), and 1 mL growth factor (FGF-B; Lonza) according to the manufacturer's protocol. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were passaged by Trypsin/EDTA (Lonza), Trypsin Neutralizing Solution (Lonza), and HEPES Buffered Saline Solution (Lonza) every 3–4 d.

Isolation from porcine eyes and labeling of photoreceptor outer segments: Retinas from freshly obtained porcine eyes were homogenized with POS buffer (115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES/KOH ph 7.5, and 1 mM dithiothreitol) containing 1.5 mM sucrose on ice. The suspension was centrifuged for 7 min at 7,510 ×*g* to sediment chunk pieces of retinas. A filter (BD Falcon, Franklin Lakes, NJ) was used to remove the deposits, and the filtrate was diluted with POS buffer and centrifuged again. The pellet was diluted with POS buffer containing 0.6 mM sucrose. The suspension was then added to the tube containing the continuous sucrose gradient and the whole was centrifuged for 90 min at 103,700 ×*g* in RP55T rotor (Hitachi Co., Ltd. Tokyo, Japan). After centrifugation, POS bands were collected and diluted with 1:3 balanced salt solution (BSS; 10 mM HEDES, 137 mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$, 1.27 mM $CaCl_2$, 0.34 mM Na_2HPO_4, and 0.44 mM KH_2PO_4). This was suspended for 7 min at 7,510 ×*g* to obtain a pure POS pellet, which was then stored in darkness at −80 °C. The supernatant was removed and the POS was taken up in several milliliters of POS buffer. The media were concentrated by centrifugation at 4,000 ×*g* using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA; molecular weight cutoff: 3,000) to combine POS with pHrodo. Unlabeled POS (5×10^7) were added to 5 mL of the BSS. POS were labeled at a final concentration of 1 mg pHrodo/10 mg protein. POS with the dye were concentrated by centrifugation at 4,000 ×*g* using the Amicon Ultra-15 centrifugal filter device (Millipore; molecular weight cutoff: 3,000) for 6 h at 4 °C [24].

Near-infrared photobiomodulation: For all experiments, we followed this protocol before each assay: ARPE-19 cells were plated at a density of 1.5×10^4 cells per well with DMEM/F-12 containing 10% FBS in 96-well plates. This was incubated for 4 d with or without 670 nm light emitting diode (LED; Sawa Denshi Kougyou, Saitama, Japan) treatment (250 s at 3.89 mW/cm^2 twice/day). The medium was changed to DMEM/F12 containing 1% FBS, and the cells were treated with an antioxidant, N-acetylcysteine (NAC; Sigma-Aldrich, St. Louis, MO) for 1 h.

Phagocytosis assays: H_2O_2 at a final concentration of 0.1 mM and 1×10^5 POS/well were added to each well and incubated for 6 h. The cells were then washed five times with 1% FBS DMEM/F-12 to allow removal of non-specific POS binding to quantify specific attachement of POS by RPE cells. Images were collected using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and then quantified using image processing software (Image-J, ver. 1.43 h; National Institutes of Health, Bethesda, MD). The area was then calculated.

Nuclear staining assay: Nuclear staining assays were conducted 6 h after H_2O_2 treatment.

Cell viability assay: Water-soluble tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay kits were used to investigate the inhibitory effect of photobiomodulation on oxidative stress-induced cytotoxicity. Briefly, 10 µl of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the cells were incubated at 37 °C for 2 h. The absorbance was measured at 492 nm (reference wavelength, 660 nm) using SkanIt Re for Varioskan Flash 2.4 (ThermoFisher Scientific Inc., Waltham, MA).

Measurement of intracellular reactive oxygen species production: The measurement of intracellular reactive oxygen species (ROS) production was estimated by $CM-H_2DCFDA$ (Invitrogen). Briefly, $CM-H_2DCFDA$ was added to the medium at a final concentration of 10 µM, followed by incubation at 37 °C for 1 h. Fluorescence was then measured using a fluorescence spectrophotometer at 488 nm excitation and 525 nm emission.

Mitochondrial membrane potential assay: The measurement of mitochondrial membrane potential was estimated using JC-1 dye (Mitochondrial Membrane Potential Probe; Invitrogen). The ARPE-19 cells (1.5×10^4 cells/well) were cultured and exposed to H_2O_2 for 6 h. The cells were washed and incubated with 10 µg/ml JC-1 at 37 °C for 15 min in the dark. Images were collected using a fluorescence microscope (BZ-9000; Keyence), which detects healthy cells with JC-1 J-aggregates (excitation/emission=540/605 nm) and unhealthy cells with mostly JC-1 monomers (excitation/emission=480/510 nm).

Western blot analysis: The ARPE-19 or hRPE cells (1.5×10^4 cells/well) were seeded onto a 24-well plate and cultured at 37 °C for 4 d. After H_2O_2 exposure, the cells were supplemented with a 1% protease inhibitor cocktail (Sigma), 1% phosphate inhibitor cocktails 2 and 3 (Sigma), and sample buffer (Wako). The lysate was centrifuged at 12,000 ×g for 10 min, and the supernatant was collected for analysis.

Protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL) with BSA as standard. An equal volume of protein sample and sample buffer with 10% 2-mercaptoethanol was electrophoresed with a 10% sodium dodecyl sulfate-polyacrylamide gel, and the separated proteins were then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The following primary antibodies were used for immunoblotting: anti-MerTK (phospho Y749 + Y 753 + Y754; ab14921) rabbit polyclonal antibody (1: 500; abcam); anti-MerTK (ab137673) rabbit polyclonal antibody; anti-phospho-FAK (Tyr397) rabbit polyclonal antibody (1: 1000; Cell Signaling Technology, Danvers, MA); anti-FAK (C-903; sc-932) rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, Inc. CA), and anti-β-actin mouse monoclonal antibody (1:5000; Sigma). An HRP-conjugated goat anti-rabbit antibody and an HRP-conjugated goat anti-mouse antibody (1:2000) were used as secondary antibodies. Band densities were measured using an imaging analyzer (LAS-4000 mini, Fujifilm, Tokyo, Japan), gel analysis software (Image Reader LAS-4000; Fujifilm), and detected band analysis software (Multi Gauge; Fujifilm).

Statistical analysis: Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using a two-tailed paired Student t test only, where $p<0.05$ indicated statistical significance.

RESULTS

Photobiomodulation enhanced the phagocytic activity in oxidative stress: To clarify the effect on phagocytic activity in near-infrared light-exposed ARPE-19 cells, the phagocytic activity in the cells was investigated after 6 h of H_2O_2 exposure by measuring the fluorescence intensity of intracellular POS. The quantification of fluorescence intensity was calculated as described in the Methods section. The fluorescence intensity was significantly reduced after H_2O_2 exposure, and NAC, an antioxidant, increased the fluorescence intensity. Moreover, photobiomodulation enhanced the fluorescence intensity of intracellular POS in comparison to the group that was only exposed to H_2O_2 (Figure 1 B,C). The group exposed to H_2O_2 and NAC group maybe also invreased the fluorescence intensity but we did not use statistical analysis between H_2O_2 only group and H_2O_2/NAC group. H_2O_2 or photobiomodulation did not affect cell death in this experimental condition (Figure 1D).

Photobiomodulation did not affect cell viability or mitochondrial membrane potential but reduced reactive oxygen species production: Cell viability was determined by WST-8 assay in order to clarify the other effects of photobiomodulation in ARPE-19 cells. In the group exposed only to H_2O_2, cell viability was reduced for 6 h compared to the control group with a reduction of 21%. Photobiomodulation did not affect cell viability (Figure 2A). CM-H2DCFH is converted to a fluorescent product (*CM-H2DCF*) when intracellular ROS are produced, was increased by H_2O_2 exposure, and 1 mM NAC significantly reduced the oxidative stress-induced ROS production in ARPE-19 cells. Daily exposure to 250 s of 670 nm photobiomodulation significantly inhibited the H_2O_2-induced ROS production (Figure 2B). To investigate the effect of photobiomodulation on mitochondrial activity, JC-1 dye was used. Neither H_2O_2 nor photobiomodulation significantly changed the red or green fluorescence (Figure 2C)

Photobiomodulation increased the expression of phosphorylated MerTk but did not change the expression of phosphorylated FAK: We investigated the mechanism of the promotion of phagocytosis by photobiomodulation. We tested changes in the levels of phagocytosis-associated proteins, FAK and MerTK, by western blot analysis after 3 h or 6 h of H_2O_2 exposure to clarify the mechanism of phagocytosis. The maximum reduction of p-FAK and p-MerTK expression was observed 3 h and 6 h after H_2O_2 exposure, respectively (data not shown). Phosphorylated FAK was significantly reduced by H_2O_2 treatment, but photobiomodulation did not change the expression of FAK in comparison to the H_2O_2-exposed group (Figure 3A). In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. Although photobiomodulation did not change the expression of phosphorylated FAK, photobiomodulation improved the reduction of phosphorylated MerTK induced by the oxidative stress (Figure 3B). NAC at 1 nM increased the expression of phosphorylated FAK and MerTK.

Photobiomodulation enhanced the phagocytic activity and increased the expression of phosphorylated MerTK: We investigated the phagocytosis activity and the expression of phosphorylated MerTK to validate our results concerning ARPE-19. The fluorescence intensity was significantly reduced after H_2O_2 exposure, and photobiomodulation enhanced the fluorescence intensity of intracellular POS in hRPE cells in comparison to the H_2O_2 only group (Figure 4A). In this point, we performed the statistical analysis between H_2O_2 only treated group and photobimodulation group. Furthermore, we investigated the expression of phosphorylated MerTK in hRPE cells. The expression of phosphorylated MerTK was increased in primary RPE cultures by photobiomodulation (Figure 4B).

DISCUSSION

In the present study, as expected, phagocytic activity was reduced by exposure to H_2O_2 (Figure 1). The activity of RPE cells is reduced by oxidative stress and the auto-oxidative lipofuscin is accumulated in the lysosomes. In addition, drusen is formed in between the RPE and Bruch's membrane. Ultimately, these things result in AMD [25,26]. The increase of oxidative stress also impairs the function of phagocytosis, and the dysfunction of phagocytosis induces the accumulation of lipofuscin [27,28].

Next, we investigated whether photobiomodulation using low-intensity and near-infrared light affects ARPE-19. Blue LED is routinely used in video display terminals and is known as an inducer of several kinds of photoreceptor cell damage in our laboratory [29]. In contrast, red LED has longer wavelengths than blue LED and has a protective effect on photoreceptors [22].

Near-infrared light photobiomodulation has a protective effect against light-induced retinal degeneration and reduced inflammation via the upregulation of mitochondrial cytochrome *c* oxidase expression in AMD mouse models [16,22]. Although photobiomodulation reduced the ROS production, it did not alter the cell viability or mitochondrial membrane potential (Figure 2). A previous report suggested that photobiomodulation has protective effects against high glucose-induced cell death of 661W cells (mouse photoreceptor cells) and retinal ganglion cells but not against high glucose-induced cell death of ARPE-19 cells [17]. This report also indicated that all cell lines, including ARPE-19, reduced the superoxide generation but did not change cytochrome *c* oxidase activity, which is related to mitochondrial activity. Furthermore, this phagocytosis assay model was set in a concentration of 0.1 mM H_2O_2, which did not change the cell death rate. Thus, low-intensity far-red light have no effects on cell viability or mitochondrial membrane potential.

Phagocytosis is related to FAK and MerTK expression. MerTK is an important protein in the ingestion of POS, and it has been shown that mutation of *MerTK* found in retinitis pigmentosa—one of the most common retinal diseases responsible for blindness—results in phagocytic dysfunction in RPE cells [30,31]. In the present study, we investigated the expression and phosphorylation of FAK and MerTK in POS exposed ARPE-19 cells to clarify the mechanism of phagocytosis enhancement with near-infrared photobiomodulation. Photobiomodulation increased phosphorylated MerTK but not phosphorylated FAK. Although photobiomodulation increased only the phosphorylated MerTK, the antioxidant NAC increased both phosphorylated FAK and MerTK. There are some difference pathway to increase the phagocytosis activity between photobiomodulation and antioxidant. A previous report suggested that mitochondrial dysfunction impairs the function of phagocytosis in retinal pigment epithelial cells [15]. Photobiomodulation may have a specific effect, which is the upregulation of phagocytosis activity through some mitochondrial pathways.

In conclusion, these findings indicate that photobiomodulation enhances phagocytosis via the MerTK-mediated upregulation of POS ingestion into RPE cells (Figure 5). Near-infrared light photobiomodulation may be a noninvasive, inexpensive, and easy adjunctive therapy to help inhibit the development of ocular diseases, such as AMD and retinitis pigmentosa. However, further experimentation and clinical studies are needed to clarify the therapeutic effects.

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