Journal of Photochemistry and Photobiology B: Biology 140 (2014) 85–93

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

# Docosahexaenoic acid aggravates photooxidative damage in retinal pigment epithelial cells via lipid peroxidation

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ARTICLE INFO

*Article history:*
Received 2 February 2014
Received in revised form 14 July 2014
Accepted 15 July 2014
Available online 1 August 2014

*Keywords:*
Retina
Retinal pigment epithelium cell
Docosahexaenoic acid
Lipid peroxidation

ABSTRACT

Docosahexaenoic acid (DHA, 22:6n-3), a long-chain polyunsaturated fatty acid (PUFA) with important functions in normal human retinal activity and vision development, is recommended to promote brain and eye development. However, recent research has revealed that increased DHA level in the retina due to linoleic acid-rich diet heightens the vulnerability of the retina to photooxidative stress. Thus, many scholars have analyzed the potential risks of DHA intake on retinal damage. This study evaluated the potential adverse effects of DHA intake on individuals usually exposed to high-light intensity conditions using a visible light-induced retinal pigment epithelium (RPE) cell damage model in vitro. Results showed that DHA promoted the proliferation of RPE cells without any cytotoxicity under dark conditions. However, DHA supplement elicited deleterious effects on RPE cells under high-intensity light conditions. That is, DHA supplement inhibited cellular proliferation, destroyed cell membrane integrity, enhanced cellular senescence, increased vascular endothelial growth factor (VEGF) release, and decreased phagocytic function. Moreover, DHA supplement increased the intracellular and extracellular levels of reactive oxygen species and the extracellular level of lipid peroxidation products under high-intensity light conditions. These results demonstrate that DHA increases the vulnerability of the retina to light damage through lipid peroxidation.



## 1. Introduction

An increasing number of people dependent on video terminal equipment, such as computer, are suffering from eye problems, such as eyestrain, blurred vision, and ocular dryness [1]. In the U.S., at least 14% of computer users have eye problems, and the annual cost of diagnosing and treating these eye problems may approach $2 billion [2,3]. Approximately 19.6%, 46.3%, and 62.5% of computer users in Japan, India, and Norway suffer from eye problems [3–5]. Overexposure to light is one of the reasons attributed to eye problems among long-time computer users [6,7].

Sufficient evidence has indicated that dietary nutritional supplements can ameliorate certain ocular diseases. Docosahexaenoic acid (DHA, 22:6n-3), a retinal long-chain polyunsaturated fatty acid (PUFA), is a nutritional supplement asserted to promote eye health. DHA is recommended by commercial advertisements and sold in health food stores. Dietary DHA is crucial for brain and eye development in infants [8]. Low plasma DHA has been linked to increased risk of poor visual and neural development in infants and children [9,10]. DHA also influences retinal structure and function in adults [11]. As a major structural lipid, DHA affects the functions of the retinal photoreceptor membrane by altering the permeability, fluidity, thickness, and lipid phase properties of this membrane [12]. However, DHA is prone to free-radical-mediated peroxidation and enzyme-catalyzed oxygenation. The retina is not only the focus of visible light but also the most metabolically active tissue; hence, the retina is always exposed to high-light and high-oxygen-pressure conditions [13]. The lipid peroxidation of DHA occurs after its absorption and transfer to the retina.

The RPE, the outermost layer of the retina, maintains the visual function of the eye. The RPE serves as a blood–retinal barrier (BRB) that transports ions, water, and metabolic end products from the subretinal space to the blood; it also absorbs nutrients such as glucose, retinol, and fatty acids from the blood and delivers these nutrients to photoreceptors [14]. RPE cells are involved in the visual cycle by reisomerizing 11-*cis*-retinal and transporting it back to photoreceptors [14]. RPE cells also release factors that elicit trophic effects on the neural retina; these effects include promoting the differentiation and survival of photoreceptors [15]. Therefore, RPE cell injury or dysfunction contributes to retinal pathologies, including

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http://dx.doi.org/10.1016/j.jphotobiol.2014.07.016
1011-1344/

monogenic retinal dystrophies, age-related macular degeneration (AMD), and retinal detachment [16,17].

The present study aims to estimate the potential adverse effects of DHA intake on individuals usually exposed to high-light intensity conditions using a visible light-induced RPE cell damage model in vitro. The absorption of visible light in RPE cells during vision was simulated, and the effects of DHA on the proliferation, senescence, and physiological functions of RPE cells were observed. This study provided insights into the dual functions of DHA in retinal physiology and pathology.

## 2. Materials and methods

### 2.1. Chemicals and reagents

DHA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Dulbecco's modified Eagle's/Ham's F12 media, blue fluorescent amine-modified microspheres, and fetal bovine serum (FBS) were purchased from Sigma–Aldrich (MO, USA). Penicillin, streptomycin, and Hanks' balanced salt solution (HBSS) were obtained from Gibco Life Technologies (Grand Island, NY). The lactic dehydrogenase (LDH) kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Senescence-associated β-galactosidase staining kit was provided by Beyotime Institute of Biotechnology (Jiangsu, China). ELISA kit was obtained from Shanghai ExCell Biology Inc. (Shanghai, China).

### 2.2. RPE cell culture

A human RPE cell line, ARPE-19 (ATCC CRL-2302) (American Type Culture Collection, Manassas, Virginia, USA), was used in the present study and cultured as previously described [7]. Cell cultures were grown in Dulbecco's modified Eagle's/Ham's F12 media (Sigma–Aldrich) supplemented with 10% FBS (Sigma–Aldrich), containing 1% antibiotic mixture of penicillin (100 U/ml) and streptomycin (100 mg/ml; Invitrogen) at 37 °C under a humidified 5% $CO_2$ atmosphere. All experiments were performed with an 80% confluent monolayer. The cells were in culture for up to four to six passages.

### 2.3. Light exposure

Light exposure experiment was performed according to our previous reports [7]. The RPE cells were subjected to white light irradiation by an integrated light-emitting diode lamp system designed by the authors [7]. The light-emitting diode lamps were purchased from Foshan Nationstar Optoelectronics Company Limited (Foshan, Guangdong, China), and the spectrum of the light was 420–800 nm according to the introduction provided by the manufacturer. Light intensity was measured with a TES-1330A light meter (TES Electrical Electronic Corporation, Taipei, Taiwan). RPE cells in an active growing state were planted into plates with serum-containing F12 medium at a concentration of $5 \times 10^5$ cells/ml. After 85% of the cells were adhered to the wall, the medium was replaced with serum-free F12 medium for control (no light exposure, no DHA treatment). For the DHA treatment groups (light exposure, DHA treatment), serum-free F12 medium containing 0–75 μmol/L of DHA was added. The RPE cells were then exposed to white light. In addition, black adhesive tape was placed on the cover of the plates in the control group to avoid exposure to light.

### 2.4. Cell proliferation assay

After subjecting to light irradiation, the cell proliferation was determined using an MTT assay. Briefly, at the end of the incubation period, the culture fluid in the wells was removed, and the cells were carefully washed once with phosphate-buffered saline (PBS) and incubated with 200 μL of a serum-free F12 medium containing 0.05% MTT in each well for 4 h. Subsequently, the culture medium was removed, and 150 μL of dimethyl sulfoxide was added to dissolve the formed formazan. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer system (Molecular Devices Co., Sunnyvale, CA).

### 2.5. Cellular membrane integrity assay

The cellular release of LDH was used as a measure of cellular damage/integrity. The enzymatic activity was determined using the LDH kit according to the manufacturer's instructions. To ensure that the absorbance of the reaction solution matched the linearity ranges (absorbance, 0.05–0.5) recommended by the manufacturer, a reasonable dilution multiple of the cellular supernate must initially be chosen.

### 2.6. VEGF detection

The amount of VEGF in the supernate of RPE culture was determined by enzyme-linked immune sorbent assay (ELISA). After light irradiation, the culture media were collected and then centrifuged at 1000×*g* for 5 min. The VEGF level in the culture medium was analyzed by a commercial VEGF ELISA kit (Shanghai ExCell Biology) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a plate reader (Molecular Devices Co.).

### 2.7. Phagocytic activity

The phagocytosis of RPE cells after light exposure was evaluated as previously described [18,19] with some modifications. Blue fluorescent amine-modified microspheres (0.05 μm) (Sigma–Aldrich) were diluted into a density of $1 \times 10^7$/mL with serum-free F12 medium and incubated at 37 °C in a humidified 5% $CO_2$ atmosphere for approximately 10 min. Subsequently, 1 mL of the medium that contains the fluorescent microspheres was added into 24-well plates after the supernatant was removed. After 24 h incubation, the phagocytic activity was determined based on the uptake level of fluorescent microspheres. The medium that contains the microspheres was removed. The adherent cells were washed twice with fresh medium to remove uningested particles before observation under a fluorescence microscope. Hank's balanced salt solution was replaced with fresh medium to wash the cells. Then, a cell suspension was prepared after digestion by pancreatin. A 200 μL aliquot of the cell suspension was transferred to black, clear-bottomed 96-well plates for fluorescence intensity analysis using a plate reader (Molecular Devices Co.) at 360 nm emission and 420 nm excitation.

### 2.8. Measurement of transepithelial electrical resistance (TER)

Fourth-passage RPE cells at a confluent density of $5 \times 10^4$ cells/cm$^2$ were placed on Transwell plates with a diameter of 6 mm and a pore size of 0.4 μm and then cultured in a medium with 10% FBS. The volumes on the apical and basolateral sides were 0.2 and 0.8 mL, respectively. The medium was changed the following day after cell attachment. After the cells reached early confluence on the third day, the serum concentration of the culture medium was reduced to 1%. The TER was measured once every 3 d using a Millicell-ERS voltmeter (Millipore Co., MA, USA) according to the manufacturer's instructions. The TER values were determined by subtracting the resistance of the filter alone (background) from the values obtained with the filters and the RPE cells. The resistance/unit area was equal to the resistance (ohm) × effective