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Combination of Lutein and Zeaxanthin, and DHA Regulated Polyunsaturated Fatty Acid Oxidation in H₂O₂-Stressed Retinal Cells

Ho Hang Leung¹ · Jean-Marie Galano² · Céline Crauste² · Thierry Durand² · Jetty Chung-Yung Lee¹ 

Abstract

Photochemical and oxidative damages in retinal pigment epithelial (RPE) cells are key events in the pathogenesis of age-related macular degeneration. Polyunsaturated fatty acids (PUFA) and carotenoids are rich in retinal cells, and under oxidative stress leads to oxidation and release lipid mediators. We evaluated the impact of carotenoids (lutein, zeaxanthin) and docosahexaenoic acid (DHA) supplementation on RPE cells under oxidative stress. ARPE-19 cells were exposed to H₂O₂ after pre-treatment with lutein, zeaxanthin, DHA, lutein + zeaxanthin or lutein + zeaxanthin with DHA. The data showed H₂O₂ reduced cell viability and DHA content, while promoted catalase activity and certain oxidized PUFA products. Treatment with DHA enhanced omega-3 PUFA enzymatic oxidation namely, anti-inflammatory mediators such as hydroxy-DHA, resolvins and neuroprotection compared to control; the effects were not influenced by the carotenoids. Omega-6 PUFA oxidation, namely pro-inflammatory HETE (5-, 9-, 12 and 20-HETE), and isoprostanes (5- and 15-F_{2t}-IsoP and 4-F_{3t}-IsoP) were reduced by lutein + zeaxanthin while the addition of DHA did not further reduce these effects. We observed transcriptional regulation of 5-lipoxygenase by DHA and GPx1 and NEFEL2 by the carotenoids that potentially resulted in decreased HETEs and glutathione respectively. 4-HNE was not affected by the treatments but 4-HHE was reduced by lutein + zeaxanthin with and without DHA. To conclude, carotenoids and DHA appeared to regulate inflammatory lipid mediators while the carotenoids also showed benefits in reducing non-enzymatic oxidation of omega-6 PUFA.

Keywords ARPE-19 · Oxidative stress · Macular degeneration · Carotenoids · DHA · Glutathione

Introduction

Age-related macular degeneration (AMD) is an ocular disease and the leading cause of visual impairment in most developed countries [1]. There are two stages of AMD, atrophic and exudative AMD, in which patients typically have atrophic AMD first and progress to exudative AMD. To date, a major risk factor for the development of AMD is

aging [2]. Other lifestyle factors such as cigarette smoking and dietary habits also contribute to the disease progression [3, 4]. Population-based clinical trials showed combination of dietary antioxidants (including vitamins, metal ions, and carotene) delays the progression of advanced AMD [5, 6]. Other epidemiological studies also suggest carotenoids i.e. lutein and zeaxanthin [7] and omega-3 polyunsaturated fatty acids (PUFAs) take part in disease protection [8, 9]. In a recent clinical study, supplementation of high dose eicosapentaenoic acid + docosahexaenoic acid (EPA + DHA) reduced AMD progression [10].

The pathophysiology of AMD is featured with gradual degradation of retinal pigment epithelial (RPE) and adjacent photoreceptor cells [11]. Oxidative and photochemical damage to the macula induces drusen formation; an extracellular debris containing modified proteins and lipids [12], which prevents nutrient exchange and create local inflammation. AMD studies use acute RPE cells (ARPE-19) to investigate molecular events [13]. A few reports focused on cellular changes in ARPE-19 upon oxidative challenge [14, 15], where G2/M phase was

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arrested and cell damage by H_2O_2 were seen, while supplementation of lutein or DHA counteracted the apoptosis. Knowing that photoreceptors and adjacent RPE cells are abundant in DHA and susceptible to oxidative stress, it is of interest to know whether oxidation products from DHA and other PUFAs contribute to the pathophysiology of AMD.

Numerous oxidized PUFA products are classified as lipid mediators that take part in modulating molecular pathways (Figs. A1, A2, Online Appendix) [16]. Those originating from lipoxygenase-mediated PUFA oxidation are regulators of inflammatory response where hydroxyeicosatetraenoic acids (HETE) derived from arachidonic acid (ARA) are pro-inflammatory and activate platelets, while DHA-derived hydroxydocosahexaenoic acids (HDHA) are anti-inflammatory with pro-resolving actions [17]. It was noted, 4-HDHA produced by 5-lipoxygenase in an *in vitro* system displayed inhibitory effect on angiogenesis, a key event associated exudative AMD pathogenesis [18].

In non-enzymatic oxidation, ARA-derived 15- F_{2t} -isoprostane (15- F_{2t} -IsoP) is not only a biomarker for oxidative stress *in vivo* but also an effective vasoconstrictor in the pathogenesis of cardiovascular diseases by activating TP receptor signalling.[16, 19, 20]. Other isoprostane groups, including F_2 -dihomo-isoprostanes (F_2 -dihomo-IsoP) from adrenic acid (AdA), F_3 -isoprostanes (F_3 -IsoP) from EPA, and F_4 -neuroprostanes (F_4 -NeuroP) from DHA are also considered biomarkers of oxidative stress-related neurological diseases. In addition, 4- F_{4t} -NeuroP showed to be beneficial in the cardiovascular system [16, 21, 22].

Genomic studies showed certain signalling pathways participate in the development of AMD [23]. Particularly, peroxisome proliferator-activated receptor (PPAR) and nuclear factor E2-related factor 2 (Nrf2) pathways are of interest. PPAR γ is known to be constitutively expressed in normal RPE cells, and tends to be up-regulated in AMD patients [24]. On the other hand, the Nrf2 pathway is implicated in AMD due to its ability to modulate multiple antioxidant enzymes [25]. Some studies have reported that therapeutic compounds of AMD act through Nrf2 pathway activation [26, 27].

In this pilot study, we investigated the potential roles of carotenoids and DHA by determining oxidized PUFA products related to antioxidant and inflammatory process, and cellular signalling in oxidative stressed ARPE-19 cells to evaluate its potential contribution to AMD development.

Materials and Methods

Chemicals and Materials

Chemicals and materials including sources and grade are described in Online Appendix. Novel oxidized PUFA compounds used for the LC-QToF-MS/MS analysis were

synthesized [28] by Institut des Biomolécules Max Mousseron group (IBMM, Montpellier, France).

ARPE-19 Cell Culture and Treatments

ARPE-19 cells were maintained in DMEM/Ham F12 containing FBS (10% v/v) and antibiotics (1% v/v) in 5% CO_2 humidified at 37 °C. The cell passage number was kept at a range of 10 – 20 to prevent significant morphological and functional modifications.

The cells were seeded in 96-well plates for 24 h before treatments and then cultured with serum-free medium containing lutein (0, 2.5, 5, 10, 20, 40 μ M), zeaxanthin (0, 2.5, 5, 10, 20, 40 μ M), and DHA (0, 10, 25, 50, 100 μ M) for 24 h. Using MTT assay, the optimal dose was selected for all treatments. The treated cells were washed with PBS (pH 7.4) and challenged with H_2O_2 (600 μ M) for 1 h prior testing. Lutein, zeaxanthin, and DHA were prepared in DMSO (0.1%). Control cells were incubated with serum-free medium containing 0.1% DMSO.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

Cell viability was determined using MTT colorimetric assay. After incubation for 4 h with MTT (0.5 mg/mL), the purple formazan produced was dissolved in DMSO. The absorbance (570 nm and 655 nm) was measured using microplate reader (BioRad 550, USA). Viable cells were expressed as percentage to control cells (100%).

Measurement of Fatty Acids and Oxidized PUFA Products

The cell samples were prepared and quantified using liquid chromatography quadrupole time-of-flight tandem mass spectrometry (LC-QToF-MS/MS) method [28] with modifications as described in Online Appendix. Fatty acids (oleic, linoleic, ARA, AdA, docosapentaenoic, EPA, DHA), isoprostanes (IsoP), HETE, HDHA, resolvins, neuroprotectin and aldehydes (as listed in Table A1 in Online Appendix) levels were determined.

Glutathione (GSH), Superoxide Dismutase (SOD), Catalase (CAT) Measurements

The treated cells were prepared and measured for GSH, SOD and CAT activities (Cayman Chemical, MI USA), and standardized with protein concentrations (BCA Protein Assay Kit, Abcam, USA). The measurement of GSH involved the enzymatic recycling of GSH [29]. The sulfhydryl group of GSH reacts with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), producing a complex of GS-TNB and 5-thio-2-nitrobenzoic

acid (TNB), monitored by spectrophotometric (Biochrom S12, UK) absorbance at 405–414 nm. The activity of SOD is frequently determined by utilizing the xanthine/xanthine oxidase system to produce superoxide anions and the formation of purple formazan from water soluble tetrazolium salt [30], monitored by the measurement of spectrophotometric absorbance at 440–460 nm. The measurement of CAT level is based on the peroxidatic activity of CAT with methanol in the presence of H₂O₂, where the formaldehyde produced is capable to bind with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) and form a bicyclic heterocycle chromogen [31, 32], detected by spectrophotometric absorbance at 540 nm.

Protein Standardization

It is common to normalize the GSH, SOD and CAT results with protein levels using bicinchoninic acid (BCA) assay. BCA assay is based on the reduction of copper (II) ions into copper (I) ions by peptide bonds in sample proteins. The chelation between BCA and copper (I) ions form a purple matrix [33] which is monitored by the absorbance at 562 nm using a spectrophotometer (Biochrom S12, UK).

Quantitative PCR

Quantitative PCR was performed using 1 µL cDNA generated by RT-PCR [28]. Applied Biosystems™ Power™ SYBR™ Green Master Mix (Thermo Fisher, USA) was used for the amplification (as listed in Table A2, Online Appendix). The reaction was carried out for 45 cycles: 60 °C for 30 s, 95 °C for 2 min, 95 °C for 15 s and 60 °C for 40 s. RNA18SN5 (Gene ID: 100,008,588) was used to normalize the expression levels. Reactions and analysis were performed on a StepOnePlus Real-Time PCR System (ABI, USA).

Statistical Analysis

Statistical analysis was performed by SPSS (version 25.0 Windows, USA). Data is expressed as mean (standard derivation). Concentrations of oxidized PUFA products, antioxidants and gene expressions between treatment groups were compared using one-way ANOVA while student t-test was applied to compare the difference within each group, with or without hydrogen peroxide. A *p*-value of 0.05 or less were considered statistically significant.

Results

DHA Increased ARPE-19 Cell Proliferation Even Under Oxidative Stress

Hydrogen peroxide (0–1000 µM) was added to the culture medium for 1 h. As shown in Fig. 1a, when the concentration reached 600 µM, the cell viability reduced to approximately 50% of the control group. Thereby, 600 µM H₂O₂ was selected for the study. The concentration of lutein, zeaxanthin or DHA treatments that retained maximum viability was also selected (Fig. 1b) where it was found to be 10 µM lutein, 10 µM zeaxanthin, and 25 µM DHA.

When the cells were treated with 10 µM lutein and 10 µM zeaxanthin and challenged with H₂O₂, viability enhanced by 20.1% and 26.5% respectively, compared to H₂O₂ alone (Fig. 1c). Treatment of 25 µM DHA increased the cell viability to near basal level (Fig. 1c). Since lutein and zeaxanthin co-exist in the retina [34] and both are associated with a lower risk of AMD [4], the synergistic effect was also tested. The cell viability and proliferation of RPE cells remained unchanged with concentrations co-treated with lutein/zeaxanthin up to 10/10 µM while 20/20 µM was cytotoxic (Fig. 1d). Addition of H₂O₂ did not reduce or increase cell proliferation when pre-treated with lutein/zeaxanthin for all concentrations. Along with the carotenoids, it was suggested DHA also take part in maintaining healthy retinal cells [35]. A combination of 10/10/12.5 µM lutein/zeaxanthin/DHA suppressed the proliferation with or without H₂O₂ challenge (Fig. 1e) whereas, higher DHA i.e., 10/10/25 µM of lutein/zeaxanthin/DHA prevented the inhibition but was unable to return to basal level (Fig. 1e).

Omega-3 PUFA was Modified by DHA Treatment

Levels of oleic acid and omega-6 PUFA namely linoleic acid, ARA and AdA were not altered by lutein, zeaxanthin or DHA treatment, even when combined. Furthermore, the challenge by H₂O₂ did not significantly change these levels, and instead, those treated with zeaxanthin significantly increased the linoleic acid concentration compared to lutein treatment (Table 1).

As anticipated, treatment of 25 µM DHA significantly increased the cellular DHA levels compared to control, lutein, zeaxanthin or lutein + zeaxanthin. DHA elevation was also seen when it was added to lutein + zeaxanthin (Table 1). In addition, docosapentaenoic acid (DPA_{n-3}) levels were augmented by DHA compared to zeaxanthin, zeaxanthin or lutein + zeaxanthin with or without DHA in the presence or absence of H₂O₂. Interestingly, EPA levels

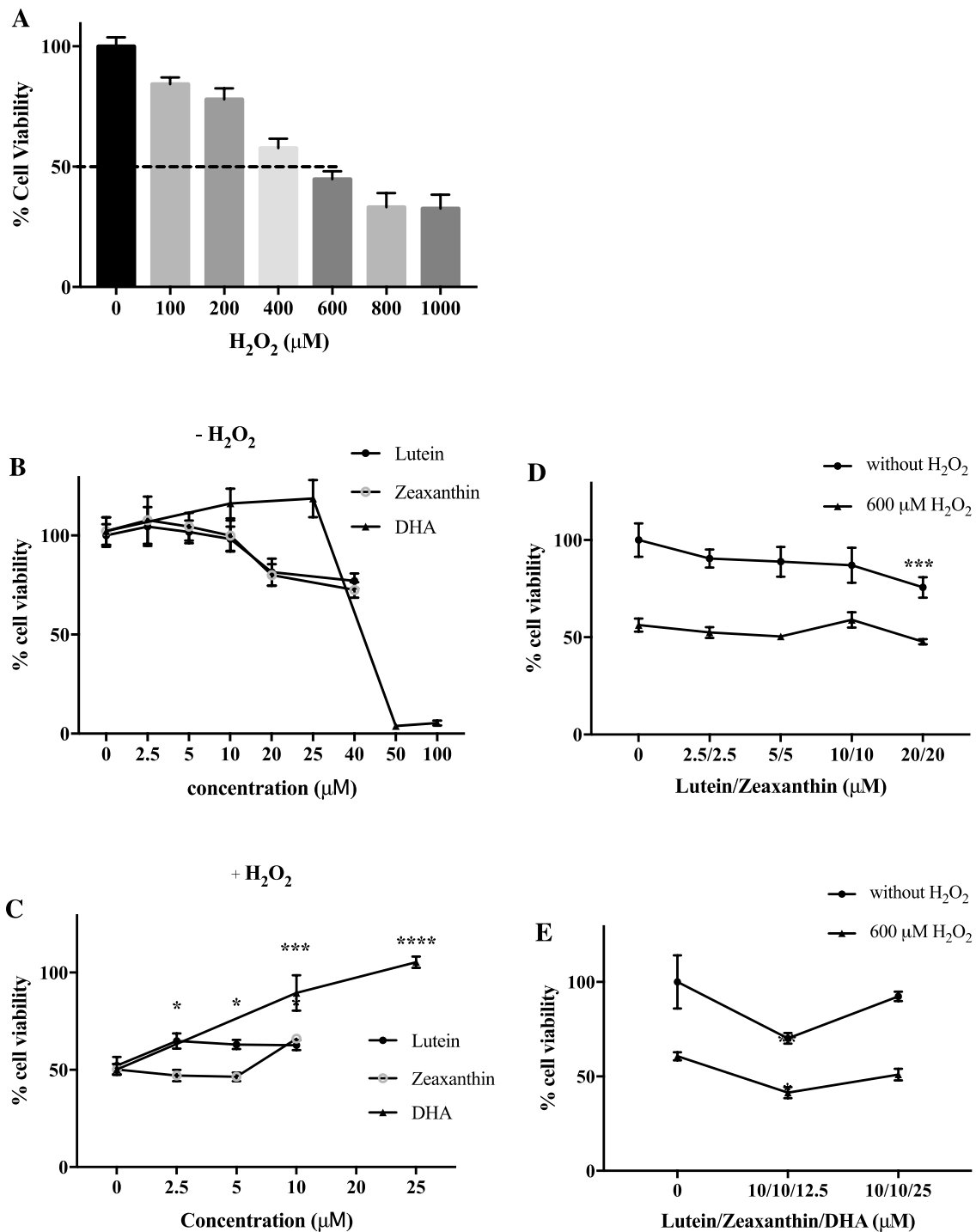


Fig. 1 Viability (%) of RPE cells (n=4) after **a** exposure to different concentrations of H₂O₂ (100 to 1000 μM) for 1 h; **b** 24 h lutein, zeaxanthin, or DHA treatment; **c** 24 h lutein, zeaxanthin, or DHA treatment and then exposed to 600 μM H₂O₂ for 1 h. *p<0.05, ***p<0.001 vs control (0 μM); **d** 24 h treatment with lutein plus

zeaxanthin (1:1) at different concentrations and then 1-h exposure with or without 600 μM H₂O₂. **p<0.01 vs control (0 μM) without H₂O₂; **e** 24 h treatment with lutein (10 μM) plus zeaxanthin (10 μM) with DHA 12.5 μM or 25 μM and then 1-h exposure with or without 600 μM H₂O₂. All values are expressed as mean (S.D.)

were enhanced by DHA treatment compared to lutein, zeaxanthin or lutein + zeaxanthin treatments and the level remained, even when challenged by H₂O₂. However, H₂O₂

challenge significantly reduced DHA levels with all the treatments (Table 1).

Table 1 Fatty acid profile of RPE cells with or without H₂O₂ challenge after lutein, zeaxanthin and DHA treatments

		Control	Lutein (L)	Zeaxanthin (Z)	DHA (D)	L+Z	L+Z+D
<i>Omega-9 monounsaturated fatty acids (MUFAs)</i>							
Oleic acid	-H ₂ O ₂	57.76 (6.35) ^a	54.43 (7.45) ^a	56.53 (7.21) ^a	55.76 (6.52) ^a	53.27 (3.93) ^a	54.33 (6.45) ^a
	+H ₂ O ₂	56.23 (9.00) ^A	54.77 (6.55) ^A	54.72 (4.95) ^A	57.59 (5.51) ^A	60.01 (6.52) ^A	53.33 (5.47) ^A
<i>Omega-6 polyunsaturated fatty acids (n-6 PUFAs)</i>							
Linoleic acid	-H ₂ O ₂	15.67 (3.35) ^a	15.20 (2.51) ^a	14.65 (2.10) ^a	15.39 (1.93) ^a	11.92 (0.36) ^a	15.58 (1.67) ^a
	+H ₂ O ₂	16.44 (5.55) ^{AB}	13.06 (2.39) ^A	18.52 (5.75) ^B	16.53 (2.88) ^{AB}	14.10 (2.86) ^{AB}	17.64 (1.63) ^{AB}
Arachidonic acid	-H ₂ O ₂	25.21 (3.64) ^a	24.39 (2.73) ^a	25.19 (2.68) ^a	24.64 (3.14) ^a	24.76 (3.40) ^a	24.21 (2.79) ^a
	+H ₂ O ₂	23.04 (1.54) ^A	24.16 (2.25) ^A	22.39 (1.05) ^A	23.76 (2.60) ^A	24.03 (1.36) ^A	23.79 (1.97) ^A
Adrenic acid	-H ₂ O ₂	5.40 (0.79) ^a	5.20 (0.65) ^a	5.54 (0.96) ^a	4.59 (0.48) ^a	4.67 (0.86) ^a	4.72 (0.83) ^a
	+H ₂ O ₂	4.96 (0.80) ^A	5.12 (0.78) ^A	6.07 (0.52) ^A	5.90 (0.65) ^A	5.30 (0.89) ^A	5.31 (0.77) ^A
<i>Omega-3 polyunsaturated fatty acids (n-3 PUFAs)</i>							
α -Linolenic acid	-H ₂ O ₂	10.15 (1.49) ^a	10.07 (2.46) ^a	9.05 (0.54) ^a	8.97 (2.92) ^a	8.25 (0.85) ^a	8.48 (2.52) ^a
	+H ₂ O ₂	9.91 (1.12) ^A	10.47 (2.53) ^A	9.18 (0.49) ^A	9.01 (2.39) ^A	8.61 (2.46) ^A	9.48 (1.61) ^A
Eicosapentaenoic acid	-H ₂ O ₂	33.40 (2.78) ^{ac}	24.26 (8.69) ^b	27.22 (3.02) ^{ab}	39.72 (6.17) ^c	25.88 (3.79) ^{ab}	35.22 (3.39) ^c
	+H ₂ O ₂	33.78 (4.89) ^{AC}	27.23 (5.00) ^{AB}	25.14 (3.99) ^B	40.23 (8.12) ^C	25.85 (3.29) ^{AB}	36.43 (3.31) ^C
Docosapentaenoic acid	-H ₂ O ₂	4.43 (1.28) ^{ac}	4.68 (1.02) ^{ac}	3.49 (0.63) ^a	7.88 (0.80) ^b	4.71 (1.38) ^{ac}	5.82 (1.94) ^c
	+H ₂ O ₂	4.10 (0.73) ^{AC}	3.80 (1.23) ^{AC}	3.04 (0.37) ^A	6.93 (1.02) ^{BC}	3.63 (1.19) ^{AC}	5.18 (0.59) ^C
Docosahexaenoic acid	-H ₂ O ₂	42.14 (3.37) ^a	40.49 (5.70) ^a	38.61 (5.66) ^a	59.42 (4.11) ^b	40.00 (4.23) ^a	64.53 (6.23) ^b
	+H ₂ O ₂	27.52 (3.84) ^{A,*}	30.96 (2.94) ^{A,*}	27.41 (1.61) ^{A,*}	40.75 (5.15) ^{B,*}	30.68 (5.71) ^{A,*}	45.47 (9.77) ^{B,*}

Values expressed in mean (S.D.) ng/million cells, n=6. Treatment concentrations were 10 μ M lutein, 10 μ M zeaxanthin and 25 μ M DHA. Different lowercase alphabets indicate statistical differences between treatment groups without hydrogen peroxide (H₂O₂) challenge; different uppercase alphabets indicate statistical differences between treatment groups with H₂O₂ challenge

*p < 0.05 statistical significance with vs without 600 μ M H₂O₂

Combination of Lutein + Zeaxanthin Reduced Omega-6 PUFA Oxidation and DHA Showed Homeostatic Control by Inducing Enzymatic Omega-3 PUFA Oxidation in the ROS-Stressed Cells

The generation of omega-6 PUFA oxidized products were limited by the carotenoids treatments with or without DHA in non-stressed cells. Surprisingly, 7-dihomo-F_{2t}-IsoP level was elevated by lutein (Table 2, Fig. A3 in Online Appendix) while lutein + zeaxanthin reduced it but not with DHA in the non-stressed cells compared to control. Treatment of DHA alone reduced 15-HETE level while lutein + zeaxanthin reduced 20-HETE and elevated 8-HETE levels in the non-stressed cells compared to control.

Exposure of H₂O₂ elevated all the oxidized products of ARA and DPA whereas only one product (7-dihomo-F_{2t}-IsoP) from AdA was elevated. Single treatment of lutein to the H₂O₂-stressed cells reduced 9- and 20-HETE levels compared to control whereas zeaxanthin only reduced 8- and 12-HETE levels. DHA treatment decreased 15-F_{2t}-IsoP, 12-HETE and 4-F_{3t}-IsoP levels but increased PGF_{2 α} of the H₂O₂-stressed cells compared to control. When the H₂O₂-stressed cells were treated with lutein + zeaxanthin, levels of 5-F_{2t}-IsoP, 15-F_{2t}-IsoP, 5-, 9- and 20-HETE were reduced and remained at similar levels even when DHA

was added (Table 2, Fig. A3 in Online Appendix). The level of 4-F_{3t}-IsoP was also lower in the H₂O₂-stressed cells compared to the control when treated with lutein + zeaxanthin with or without DHA. HNE levels were elevated in H₂O₂-stressed cells and were unaffected by all the treatments.

Interestingly, when lutein and zeaxanthin was added separately, 17-HDHA level was reduced in the non-stressed cells compared to control (Table 3, Fig. A4 in Online Appendix). However, DHA alone elevated RvD1, NPD1, 7-HDHA and 14-F_{3t}-IsoP levels. When the non-stressed cells were treated with lutein + zeaxanthin, 8-F_{3t}-IsoP and 10-F_{4t}-NeuroP levels were elevated while NPD1, 17-HDHA and 14-F_{3t}-IsoP were reduced. Adding DHA to lutein + zeaxanthin enhanced RvD1, NPD1 and 4-, 8-, 11- and 17-HDHA levels while 14-F_{3t}-IsoP level was reduced compared to control.

The challenge of H₂O₂ enhanced omega-3 PUFA oxidation (Table 3, Fig. A4 in Online Appendix) where levels of 5-F_{3t}-IsoP, 4-, 10- and 20-F_{4t}-NeuroP, 7-, 8-, 14-, 17-HDHA, 14-F_{3t}-IsoP and HHE were induced compared to control. Single treatment of lutein reduced 4-F_{4t}-NeuroP, 17-HDHA and 14-F_{3t}-IsoP levels while zeaxanthin alone reduced 5-F_{3t}- and 18-F_{3t}-IsoP, 4-F_{4t}-NeuroP, 14- and 17-HDHA, 14-F_{3t}-IsoP and HHE levels in the H₂O₂-stressed cells compared to control. However, DHA treatment elevated 8-F_{3t}-IsoP,

Table 2 Lipid oxidation products of omega-6 PUFAs measured in RPE cells with or without H₂O₂ challenge after lutein, zeaxanthin and DHA treatments

		Control	Lutein (L)	Zeaxanthin (Z)	DHA (D)	L+Z	L+Z+D
<i>Arachidonic acid derived</i>							
5-F _{2t} -IsoP	-H ₂ O ₂	5.61 (1.80) ^a	4.46 (1.90) ^a	4.78 (2.01) ^a	6.85 (1.97) ^a	4.29 (0.81) ^a	5.19 (0.66) ^a
	+H ₂ O ₂	15.90 (2.06) ^{A,*}	13.38 (2.31) ^{A,*}	13.71 (3.68) ^{A,*}	13.62 (2.33) ^{A,*}	7.44 (1.71) ^{B,*}	7.53 (0.96) ^B
15-F _{2t} -IsoP	-H ₂ O ₂	0.57 (0.15) ^a	0.54 (0.09) ^a	0.43 (0.13) ^a	0.46 (0.18) ^a	0.43 (0.18) ^a	0.48 (0.25) ^a
	+H ₂ O ₂	1.52 (0.26) ^{A,*}	1.16 (0.40) ^{AB,*}	1.14 (0.28) ^{AB,*}	0.92 (0.32) ^{BC,*}	0.75 (0.29) ^{BC}	0.69 (0.40) ^C
PGF _{2α}	-H ₂ O ₂	1.48 (0.43) ^a	1.07 (0.51) ^a	1.33 (0.13) ^a	1.84 (0.77) ^a	1.43 (0.13) ^a	1.31 (0.08) ^a
	+H ₂ O ₂	2.13 (0.89) ^{AB}	1.97 (1.18) ^{AB}	1.43 (0.22) ^A	2.96 (0.99) ^B	1.53 (0.27) ^A	1.40 (0.21) ^A
5-HETE	-H ₂ O ₂	0.73 (0.35) ^a	0.84 (0.45) ^a	0.87 (0.15) ^a	1.28 (0.47) ^a	0.74 (0.27) ^a	0.32 (0.14) ^a
	+H ₂ O ₂	1.54 (0.46) ^{ABC}	1.31 (0.61) ^{AC}	1.76 (1.22) ^{AB}	2.50 (1.38) ^{B,*}	0.57 (0.08) ^C	0.66 (0.36) ^C
8-HETE	-H ₂ O ₂	0.75 (0.31) ^{ab}	0.64 (0.22) ^{ab}	0.59 (0.24) ^a	0.91 (0.27) ^{ab}	1.55 (0.52) ^b	0.64 (0.15) ^{ab}
	+H ₂ O ₂	2.06 (1.02) ^{A,*}	1.74 (0.50) ^{AB,*}	1.07 (0.53) ^B	1.58 (0.94) ^{AB,*}	2.14 (0.48) ^A	1.72 (0.44) ^{AB}
9-HETE	-H ₂ O ₂	2.08 (0.98) ^a	1.07 (0.37) ^a	1.92 (0.43) ^a	2.45 (1.34) ^a	1.63 (0.17) ^a	1.59 (0.43) ^a
	+H ₂ O ₂	4.82 (1.68) ^{A,*}	2.57 (1.36) ^B	4.82 (1.51) ^{A,*}	6.03 (2.26) ^{A,*}	1.31 (0.14) ^B	1.60 (0.36) ^B
11-HETE	-H ₂ O ₂	0.78 (0.35) ^a	0.58 (0.21) ^a	0.41 (0.15) ^a	0.47 (0.27) ^a	0.31 (0.13) ^a	0.59 (0.30) ^a
	+H ₂ O ₂	1.54 (0.68) ^{A,*}	0.97 (0.65) ^{A,*}	1.57 (0.65) ^{A,*}	1.52 (0.32) ^{A,*}	1.24 (0.05) ^{A,*}	1.39 (0.24) ^{A,*}
12-HETE	-H ₂ O ₂	1.64 (0.27) ^a	1.54 (0.20) ^a	1.37 (0.42) ^a	1.32 (1.13) ^a	1.26 (0.10) ^a	1.40 (0.30) ^a
	+H ₂ O ₂	3.35 (0.83) ^{A,*}	2.35 (0.85) ^B	1.84 (0.45) ^{BC}	1.80 (0.86) ^{BC}	1.33 (0.32) ^C	1.38 (0.14) ^{BC}
15-HETE	-H ₂ O ₂	5.48 (0.78) ^{ab}	5.82 (0.83) ^a	5.78 (0.50) ^a	4.06 (0.91) ^b	4.72 (0.91) ^{ab}	4.16 (0.58) ^b
	+H ₂ O ₂	5.14 (1.15) ^A	5.13 (0.96) ^A	5.56 (1.25) ^A	5.40 (1.09) ^{A,*}	4.12 (0.40) ^A	4.25 (0.23) ^A
20-HETE	-H ₂ O ₂	3.24 (0.99) ^{ab}	2.52 (0.86) ^{ab}	2.64 (0.43) ^{ab}	3.88 (0.92) ^a	1.87 (0.41) ^b	3.00 (0.49) ^{ab}
	+H ₂ O ₂	5.78 (0.82) ^{A,*}	5.30 (0.90) ^{AB,*}	4.97 (1.01) ^{ABC,*}	6.02 (0.87) ^{A,*}	3.93 (0.94) ^{BC,*}	3.54 (1.20) ^C
<i>Adrenic acid derived</i>							
7-Dihomo-F _{2t} -IsoP	-H ₂ O ₂	5.71 (1.65) ^{ab}	8.26 (1.82) ^b	7.40 (2.06) ^{ab}	6.50 (2.35) ^{ab}	4.67 (2.10) ^{ac}	2.72 (1.19) ^c
	+H ₂ O ₂	15.13 (1.23) ^{AB,*}	15.50 (1.65) ^{B,*}	14.23 (1.36) ^{AB,*}	13.20 (2.54) ^{AB,*}	13.48 (1.14) ^{A,*}	12.40 (1.36) ^{AB,*}
17-Dihomo-F _{2t} -IsoP	-H ₂ O ₂	0.67 (0.35) ^a	0.58 (0.22) ^a	0.62 (0.32) ^a	0.85 (0.58) ^a	0.82 (0.48) ^a	0.72 (0.24) ^a
	+H ₂ O ₂	0.78 (0.37) ^A	0.66 (0.33) ^A	0.91 (0.58) ^A	0.96 (0.85) ^A	0.76 (0.37) ^A	1.04 (0.54) ^A
7-Dihomo-F _{2t} -IsoF	-H ₂ O ₂	8.42 (3.39) ^a	8.24 (4.73) ^a	8.71 (5.36) ^a	6.96 (3.28) ^a	7.21 (3.34) ^a	8.32 (3.47) ^a
	+H ₂ O ₂	5.69 (4.88) ^A	5.07 (3.70) ^A	5.61 (5.34) ^A	5.99 (4.19) ^A	6.11 (4.16) ^A	6.56 (4.48) ^A
17-Dihomo-F _{2t} -IsoF	-H ₂ O ₂	1.54 (0.87) ^a	2.11 (1.88) ^a	2.21 (1.73) ^a	2.54 (3.01) ^a	2.31 (2.55) ^a	2.73 (2.66) ^a
	+H ₂ O ₂	1.66 (2.32) ^A	1.99 (2.64) ^A	1.65 (2.26) ^A	1.39 (1.73) ^A	1.40 (1.44) ^A	2.00 (2.79) ^A
<i>Omega-6 Docosapentaenoic acid derived</i>							
4-F _{3t} -IsoP	-H ₂ O ₂	1.16 (0.50) ^a	1.92 (0.68) ^a	1.61 (0.51) ^a	1.42 (0.45) ^a	1.62 (0.40) ^a	0.88 (0.33) ^a
	+H ₂ O ₂	3.94 (1.48) ^{A,*}	3.41 (0.87) ^{AB,*}	3.24 (1.20) ^{AB,*}	2.36 (0.39) ^B	2.85 (0.70) ^{A,*}	1.79 (0.90) ^C
<i>Peroxidation end-product</i>							
4-HNE	-H ₂ O ₂	5.94 (1.19) ^a	6.69 (1.13) ^a	6.83 (1.48) ^a	5.92 (1.28) ^a	6.42 (0.99) ^a	6.63 (0.71) ^a
	+H ₂ O ₂	8.77 (1.96) ^{A,*}	9.03 (1.46) ^{A,*}	8.37 (0.81) ^{A,*}	8.47 (0.71) ^{A,*}	8.59 (0.45) ^{A,*}	9.18 (0.61) ^{A,*}

Values expressed in mean (S.D.) ng/million cells, n=6. Treatment concentrations were 10 μM lutein, 10 μM zeaxanthin and 25 μM DHA. Different lower-case alphabets indicate statistical differences between treatment groups without hydrogen peroxide (H₂O₂) challenge; different uppercase capitals indicate statistical differences between treatment groups with H₂O₂ challenge

*p < 0.05 statistical significance with vs without 600 μM H₂O₂

10-F_{4t}-NeuroP, 4-, 7- and 11-HDHA and NPD1 levels but reduced 4-F_{4t}-NeuroP and 14-F_{3t}-IsoP levels compared to control. It was surprising to observe numerous amounts of omega-3 PUFA oxidized products to be reduced by lutein + zeaxanthin namely, 18-F_{3t}-IsoP, 4- and 20-F_{4t}-NeuroP, 8-, 11- and 17-HDHA, NPD1, 14-F_{3t}-IsoP and HHE

levels in the H₂O₂-stressed cells compared to control while only 8-F_{3t}-IsoP level was elevated. The addition of DHA to lutein + zeaxanthin did not further reduce the levels of 18-F_{3t}-IsoP, 4- and 20-F_{4t}-NeuroP, 14-F_{3t}-IsoP and HHE but it elevated RvE1, RvD1, NPD1 and 4-, 7-, 8- and 11-HDHA levels.

Table 3 Lipid oxidation products of omega-3 PUFAs measured in RPE cells with or without H₂O₂ challenge after lutein, zeaxanthin and DHA treatments

		Control	Lutein (L)	Zeaxanthin (Z)	DHA (D)	L+Z	L+Z+D
<i>Eicosapentaenoic acid derived</i>							
5-F _{3t} -IsoP	-H ₂ O ₂	3.39 (0.74) ^a	3.05 (1.23) ^a	0.93 (0.59) ^a	2.78 (0.88) ^a	1.45 (1.02) ^a	1.90 (0.84) ^a
	+H ₂ O ₂	6.16 (1.29) ^{AC,*}	6.12 (1.84) ^{AC,*}	2.45 (0.52) ^B	5.18 (0.90) ^{AC,*}	5.81 (3.24) ^{C,*}	6.16 (1.97) ^{C,*}
8-F _{3t} -IsoP	-H ₂ O ₂	1.06 (0.40) ^a	1.29 (0.48) ^{ab}	0.84 (0.14) ^a	1.62 (0.44) ^{ab}	2.12 (0.84) ^b	1.67 (0.27) ^a
	+H ₂ O ₂	1.69 (0.75) ^A	2.15 (0.37) ^{AB,*}	1.12 (0.35) ^A	2.46 (0.80) ^{B,*}	3.63 (0.36) ^{C,*}	2.89 (0.63) ^{BC,*}
18-F _{3t} -IsoP	-H ₂ O ₂	1.83 (0.83) ^a	2.12 (0.80) ^a	1.65 (0.20) ^a	2.21 (1.04) ^a	1.19 (0.61) ^a	1.07 (0.23) ^a
	+H ₂ O ₂	4.50 (2.63) ^A	4.05 (1.06) ^A	2.14 (0.68) ^{BC}	3.80 (1.46) ^{AB}	1.10 (0.32) ^C	0.90 (0.42) ^C
RvE1	-H ₂ O ₂	1.39 (0.13) ^a	1.46 (0.16) ^a	1.34 (0.11) ^a	1.46 (0.32) ^a	1.39 (0.19) ^a	1.59 (0.30) ^a
	+H ₂ O ₂	1.39 (0.16) ^A	1.51 (0.39) ^A	1.31 (0.06) ^A	1.74 (0.44) ^A	1.41 (0.29) ^A	2.25 (0.52) ^{B,*}
<i>Docosahexaenoic acid derived</i>							
4-F _{4t} -NeuroP	-H ₂ O ₂	3.05 (1.15) ^a	2.31 (0.86) ^a	2.34 (1.02) ^a	2.72 (0.76) ^a	1.86 (0.39) ^a	1.38 (0.27) ^a
	+H ₂ O ₂	8.43 (1.09) ^{A,*}	5.36 (1.21) ^{B,*}	4.99 (1.14) ^{B,*}	5.56 (1.74) ^{B,*}	2.62 (1.40) ^C	2.57 (1.71) ^C
10-F _{4t} -NeuroP	-H ₂ O ₂	7.06 (2.75) ^a	10.97 (2.89) ^{ab}	7.91 (2.72) ^{ac}	12.88 (1.71) ^{bc}	13.25 (1.62) ^b	9.32 (0.95) ^a
	+H ₂ O ₂	13.52 (2.63) ^{A,*}	14.87 (3.73) ^A	16.33 (3.43) ^{AB,*}	21.54 (4.97) ^{B,*}	16.94 (3.81) ^{AB}	14.80 (3.98) ^{A,*}
13-F _{4t} -NeuroP	-H ₂ O ₂	1.10 (0.56) ^a	2.09 (0.78) ^a	1.62 (0.24) ^a	1.44 (0.40) ^a	1.79 (0.21) ^a	1.52 (0.24) ^a
	+H ₂ O ₂	3.08 (2.19) ^A	3.75 (1.89) ^A	3.96 (2.89) ^{A,*}	4.43 (1.28) ^{A,*}	2.46 (0.70) ^A	1.92 (0.77) ^A
14-F _{4t} -NeuroP	-H ₂ O ₂	3.77 (2.18) ^a	3.33 (1.31) ^a	2.86 (0.44) ^a	4.70 (2.32) ^a	4.84 (1.18) ^a	4.88 (2.09) ^a
	+H ₂ O ₂	5.39 (3.33) ^{AB}	4.21 (2.43) ^{AB}	2.98 (0.51) ^A	4.77 (1.04) ^{AB}	4.99 (1.19) ^{AB}	6.52 (2.23) ^B
20-F _{4t} -NeuroP	-H ₂ O ₂	1.33 (0.35) ^a	1.73 (0.86) ^a	1.52 (0.12) ^a	2.63 (1.36) ^a	2.18 (0.93) ^a	1.34 (0.11) ^a
	+H ₂ O ₂	4.95 (2.15) ^{A,*}	3.95 (2.38) ^{AB,*}	1.90 (0.54) ^{BC}	4.61 (2.14) ^A	2.00 (0.75) ^{BC}	1.61 (0.47) ^C
RvD1	-H ₂ O ₂	1.48 (0.17) ^{ab}	1.48 (0.19) ^{ab}	1.13 (0.04) ^a	1.90 (0.18) ^{bc}	1.52 (0.20) ^{ab}	2.03 (0.58) ^c
	+H ₂ O ₂	1.51 (0.14) ^{AB}	1.45 (0.23) ^{AB}	1.14 (0.07) ^A	2.59 (0.20) ^{C,*}	1.78 (0.64) ^B	2.42 (0.24) ^{C,*}
NPD1	-H ₂ O ₂	1.79 (0.16) ^a	1.62 (0.19) ^{ac}	1.61 (0.18) ^{ac}	2.40 (0.22) ^b	1.20 (0.07) ^c	2.40 (0.35) ^b
	+H ₂ O ₂	1.74 (0.34) ^{AC}	1.95 (0.32) ^A	1.69 (0.35) ^{AC}	3.58 (0.32) ^{B,*}	1.38 (0.10) ^C	3.82 (0.60) ^{B,*}
4-HDHA	-H ₂ O ₂	0.92 (0.59) ^a	0.67 (0.10) ^a	0.42 (0.17) ^a	1.21 (0.57) ^a	0.68 (0.43) ^a	2.50 (0.66) ^b
	+H ₂ O ₂	1.46 (0.33) ^A	0.96 (0.49) ^A	0.92 (0.44) ^A	4.23 (1.05) ^{B,*}	1.39 (0.47) ^A	4.26 (0.46) ^{B,*}
7-HDHA	-H ₂ O ₂	1.34 (0.24) ^a	1.73 (0.51) ^{ab}	1.27 (0.16) ^a	2.25 (0.45) ^{bc}	1.15 (0.07) ^a	2.11 (0.53) ^{ac}
	+H ₂ O ₂	2.21 (0.26) ^{A,*}	2.89 (0.98) ^{AB,*}	2.39 (0.47) ^{A,*}	3.71 (0.62) ^{BC,*}	1.40 (0.15) ^A	3.97 (0.65) ^{C,*}
8-HDHA	-H ₂ O ₂	2.25 (0.36) ^a	1.91 (0.11) ^a	2.30 (0.40) ^a	2.67 (0.21) ^a	1.65 (0.19) ^a	3.29 (0.69) ^b
	+H ₂ O ₂	3.41 (0.83) ^{AC,*}	4.09 (0.48) ^{AB,*}	3.06 (0.57) ^C	3.82 (0.53) ^{ABC,*}	2.92 (0.36) ^{C,*}	4.53 (1.13) ^{B,*}
11-HDHA	-H ₂ O ₂	1.48 (0.26) ^a	1.42 (0.18) ^a	1.52 (0.30) ^a	1.30 (0.14) ^a	1.55 (0.18) ^a	2.27 (0.55) ^b
	+H ₂ O ₂	1.70 (0.23) ^{AC}	1.85 (0.54) ^{AC}	1.45 (0.43) ^A	2.11 (0.63) ^{C,*}	1.19 (0.07) ^A	2.90 (0.47) ^{B,*}
14-HDHA	-H ₂ O ₂	2.41 (0.22) ^a	2.65 (0.28) ^a	2.57 (0.20) ^a	2.53 (0.29) ^a	2.15 (0.05) ^a	2.42 (0.19) ^a
	+H ₂ O ₂	3.69 (0.48) ^{A,*}	3.53 (0.55) ^{A,*}	2.72 (0.43) ^B	3.57 (0.34) ^{A,*}	2.58 (0.24) ^B	4.13 (1.02) ^{A,*}
17-HDHA	-H ₂ O ₂	2.74 (0.39) ^a	1.60 (0.22) ^b	1.57 (0.35) ^b	3.15 (0.42) ^{ac}	1.54 (0.59) ^b	4.00 (0.71) ^c
	+H ₂ O ₂	3.84 (0.73) ^{A,*}	1.99 (0.58) ^B	1.61 (0.38) ^B	3.79 (0.69) ^A	2.03 (0.60) ^B	4.52 (0.82) ^A

Table 3 (continued)

	Control	Lutein (L)	Zeaxanthin (Z)	DHA (D)	L+Z	L+Z+D
<i>Omega-3 Docosapentaenoic acid derived</i>						
14-F _{3t} -IsoP	2.27 (1.09) ^a 5.29 (0.92) ^{A,*}	1.51 (0.40) ^{ab} 4.04 (0.44) ^{B,*}	1.13 (0.43) ^{bc} 0.96 (0.65) ^C	1.05 (0.66) ^{bc} 1.79 (0.78) ^C	0.69 (0.38) ^{bc} 0.82 (0.31) ^C	0.43 (0.16) ^C 0.77 (0.68) ^C
<i>Peroxidation end-product</i>						
4-HHE	0.98 (0.42) ^a 1.97 (0.72) ^{A,*}	0.98 (0.41) ^a 1.44 (0.32) ^{AB}	0.75 (0.13) ^a 1.14 (0.60) ^{BC}	0.92 (0.26) ^a 1.67 (0.74) ^{AB,*}	0.64 (0.31) ^a 0.62 (0.18) ^{CD}	0.60 (0.09) ^a 0.58 (0.13) ^{CD}

Values expressed in mean (S.D.) ng/million cells, n=6. Treatment concentrations were 10 μ M lutein, 10 μ M zeaxanthin and 25 μ M DHA. Different lower-case alphabets indicate statistical differences between treatment groups without hydrogen peroxide (H₂O₂) challenge; different uppercase capitals indicate statistical differences between treatment groups with H₂O₂ challenge. * p < 0.05 statistical significance with vs without 600 μ M H₂O₂

Limited Gene Expressions of Lipid Metabolism and Antioxidant Response by Lutein, Zeaxanthin and DHA

Unexpectedly, lutein, zeaxanthin or DHA treatments with or without H₂O₂ challenge did not manipulate cellular gene expression associated with lipid metabolism and antioxidant response (Table 4). However, H₂O₂ challenge to lutein + zeaxanthin treated RPE cells down-regulated the expressions of PPAR γ , ALOX-5, NFE2L2, and GPx-1 compared to the control group. These expressions were not altered when DHA was added in the treatment. CAT activity was induced by H₂O₂ challenge but were not affected by lutein, zeaxanthin, DHA or lutein + zeaxanthin with or without DHA (Fig. 2). Moreover, SOD levels were not induced by H₂O₂ challenge and showed no change by the treatments. However, DHA alone augmented GSH levels regardless of H₂O₂ challenge compared to control and the effect remained even with lutein + zeaxanthin. Treatment of lutein + zeaxanthin alone with H₂O₂ showed increased GSH compared to control.

Discussion

The findings from this study addressed some important functions of lutein + zeaxanthin and DHA in the oxidation pathway of PUFA in RPE cells. Lutein or zeaxanthin or when combined displayed potent antioxidant properties in non-enzymatic oxidation process for both omega-6 and omega-3 PUFA where consistent decreased levels of isoprostanes (namely 5-F_{2t}- and 15-F_{2t}-IsoP and 4-F_{4t}-NeuroP) and HHE were observed. The incorporation of DHA alone or with lutein + zeaxanthin to the H₂O₂-stressed cells showed homeostatic control where it enhanced enzymatic oxidation and subsequently up-regulated specialized pro-resolving mediators such as RvD1 and NPD1 that potentially compensated the loss of 17-HDHA by lutein and/or zeaxanthin. These observation further supports our recent findings in human study, where poor circulatory lutein + zeaxanthin and DHA are associated to AMD risk in the expense of elevated omega-6 PUFA oxidation and loss of omega-3 PUFA oxidation [35].

The yellow macula is rich in carotenoid pigments mainly, lutein and zeaxanthin. They are suggested to protect the macula from photo-oxidative damage by filtering blue light and act as antioxidant to quench the excited triplet molecules or singlet oxygen that can induce reactive oxygen species (ROS) such as lipid peroxides or superoxide anions [36]. In addition, lutein and zeaxanthin can indirectly protect the RPE by absorbing short-wavelength light; it is normally taken up by lipofuscin, accumulate in the RPE and generate ROS via photosensitization. Moreover, DHA

Table 4 Cellular gene expression of RPE cell with or without H₂O₂ challenge after lutein, zeaxanthin and DHA treatments

		Control	Lutein (L)	Zeaxanthin (Z)	DHA (D)	L+Z	L+Z+D
<i>Lipid metabolism</i>							
PPAR α	–H ₂ O ₂	1.00 (0.26) ^a	1.13 (0.49) ^a	0.96 (0.07) ^a	1.07 (0.34) ^a	0.86 (0.54) ^a	0.82 (0.41) ^a
	+H ₂ O ₂	1.57 (0.60) ^A	0.90 (0.02) ^A	1.11 (0.77) ^A	0.90 (0.30) ^A	1.07 (0.31) ^A	0.90 (0.44) ^A
PPAR δ	–H ₂ O ₂	1.00 (0.25) ^a	1.20 (0.37) ^a	1.19 (0.02) ^a	0.83 (0.09) ^a	1.26 (0.68) ^a	1.40 (0.24) ^a
	+H ₂ O ₂	1.43 (0.69) ^A	1.87 (0.18) ^A	1.87 (0.55) ^A	1.45 (0.65) ^A	1.33 (0.25) ^A	1.54 (0.15) ^A
PPAR γ	–H ₂ O ₂	1.00 (0.16) ^a	0.97 (0.18) ^a	0.89 (0.40) ^a	1.26 (0.35) ^a	0.86 (0.31) ^a	1.12 (0.08) ^a
	+H ₂ O ₂	1.76 (0.63) ^A	1.67 (0.51) ^{AB}	1.22 (0.23) ^{AB}	1.46 (0.19) ^{AB}	1.61 (0.57) ^{AB}	0.84 (0.21) ^B
ALOX-5	–H ₂ O ₂	1.00 (0.25) ^a	0.49 (0.26) ^a	0.76 (0.28) ^a	0.71 (0.43) ^a	0.81 (0.39) ^a	0.63 (0.22) ^a
	+H ₂ O ₂	1.69 (0.60) ^A	0.98 (0.44) ^{AB}	0.80 (0.33) ^{AB}	0.84 (0.44) ^{AB}	1.39 (0.62) ^A	0.51 (0.06) ^B
ALOX-12	–H ₂ O ₂	1.00 (0.31) ^a	0.83 (0.43) ^a	1.38 (0.28) ^a	0.55 (0.04) ^a	0.95 (0.24) ^a	1.33 (0.39) ^a
	+H ₂ O ₂	1.24 (0.94) ^A	0.81 (0.15) ^A	1.13 (0.04) ^A	1.07 (0.75) ^A	1.07 (0.48) ^A	1.24 (0.26) ^A
<i>Antioxidant response</i>							
NFE2L2	–H ₂ O ₂	1.00 (0.15) ^a	1.00 (0.17) ^a	0.97 (0.16) ^a	0.93 (0.29) ^a	0.69 (0.03) ^a	0.76 (0.08) ^a
	+H ₂ O ₂	1.35 (0.37) ^A	1.11 (0.23) ^{AB}	0.99 (0.04) ^{AB}	0.98 (0.17) ^{AB}	0.81 (0.11) ^B	0.77 (0.14) ^B
Keap-1	–H ₂ O ₂	1.00 (0.18) ^a	0.94 (0.30) ^a	0.92 (0.23) ^a	0.99 (0.09) ^a	0.93 (0.54) ^a	0.81 (0.11) ^a
	+H ₂ O ₂	0.87 (0.40) ^A	1.04 (0.30) ^A	0.91 (0.26) ^A	0.96 (0.42) ^A	0.84 (0.25) ^A	0.78 (0.02) ^A
GPx-1	–H ₂ O ₂	1.00 (0.16) ^a	0.99 (0.09) ^a	0.76 (0.17) ^a	1.03 (0.15) ^a	0.70 (0.37) ^a	0.70 (0.10) ^a
	+H ₂ O ₂	1.40 (0.47) ^A	1.35 (0.20) ^A	1.09 (0.03) ^{AB}	1.17 (0.65) ^{AB}	0.68 (0.16) ^B	0.59 (0.04) ^B
GPx-4	–H ₂ O ₂	1.00 (0.18) ^a	0.50 (0.05) ^a	0.92 (0.11) ^a	0.73 (0.29) ^a	0.90 (0.23) ^a	0.72 (0.07) ^a
	+H ₂ O ₂	1.01 (0.30) ^A	1.03 (0.10) ^A	0.85 (0.30) ^A	0.97 (0.51) ^A	0.94 (0.23) ^A	0.67 (0.21) ^A
SOD-1	–H ₂ O ₂	1.00 (0.21) ^a	0.82 (0.34) ^a	0.81 (0.23) ^a	0.98 (0.08) ^a	1.17 (0.41) ^a	0.55 (0.19) ^a
	+H ₂ O ₂	1.17 (0.37) ^A	1.22 (0.20) ^A	0.83 (0.12) ^A	1.21 (0.39) ^A	1.32 (0.37) ^A	0.69 (0.16) ^A
<i>Immune response</i>							
NF- κ B	–H ₂ O ₂	1.00 (0.15) ^a	1.43 (0.17) ^a	1.09 (0.19) ^a	1.22 (0.45) ^a	1.15 (0.32) ^a	1.32 (0.46) ^a
	+H ₂ O ₂	1.35 (0.24) ^A	1.39 (0.38) ^A	1.67 (0.53) ^A	1.26 (0.22) ^A	1.23 (0.42) ^A	1.29 (0.53) ^A
IL-6	–H ₂ O ₂	1.00 (0.20) ^a	1.20 (0.43) ^a	1.44 (0.48) ^a	1.05 (0.49) ^a	1.06 (0.26) ^a	0.96 (0.35) ^a
	+H ₂ O ₂	1.37 (0.21) ^A	1.77 (0.54) ^A	1.42 (0.31) ^A	1.49 (0.65) ^A	1.54 (0.67) ^A	1.04 (0.67) ^A

Values expressed in mean (S.D.), n=3. Treatment concentrations were 10 μ M lutein, 10 μ M zeaxanthin and 25 μ M DHA. Different lower-case alphabets indicate statistical differences between treatment groups without hydrogen peroxide (H₂O₂) challenge; different uppercase alphabets indicate statistical differences between treatment groups with H₂O₂ challenge

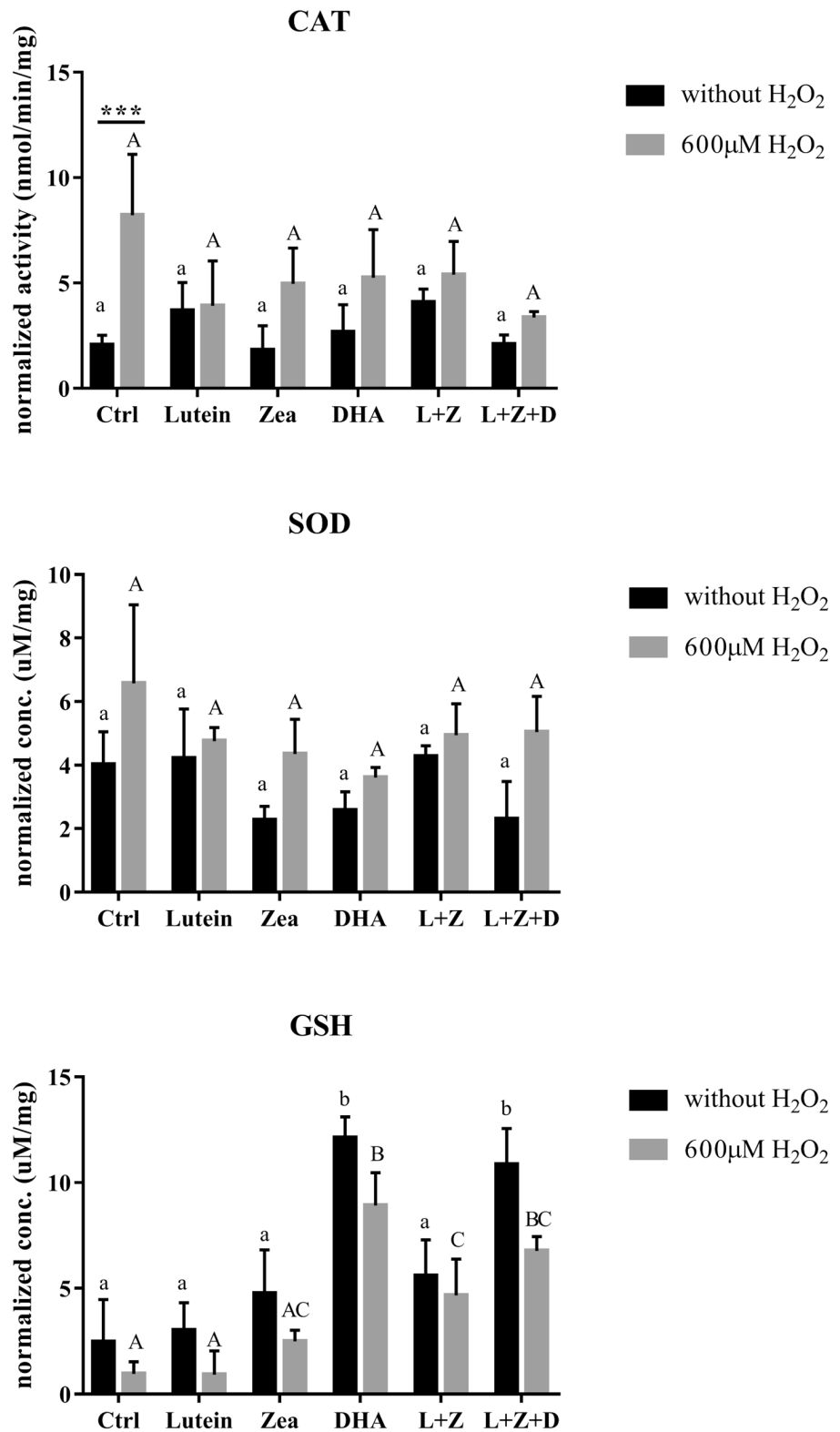
is predominantly present in the macula and modulates the survival of photoreceptor cells to maintain normal vision [35]. The presence of both carotenoids and DHA in the macula coherently indicates its vital functional properties in oxidative stressed RPE cells.

Elevated ROS are implicated in the pathogenesis of numerous neurodegenerative diseases, including AMD. To withstand the high load of photons and ROS, retinal cells are equipped with endogenous antioxidants to prevent photo-oxidative damage and maintain homeostasis. We demonstrated that a drastic elevation of ROS such as H₂O₂ in RPE will modify cell viability and activate CAT but not GSH or SOD. It appears antioxidant modulation to be post-transcriptional as shown by NFE2L2 and ALOX-5. The endogenous antioxidant enzymes were insufficient to reduce intracellular ROS accumulation, resulting in DHA depletion and subsequent oxidation. In addition, epidemiological studies consistently showed low circulating DHA to be highly

correlated with risk of developing AMD [4, 37]. Moreover, in both animal and cell models, the deficiency of omega-3 PUFAs in photoreceptors was related to the development of AMD [15, 38]. Taken together, our results indicate the treatments were insufficient to regulate the antioxidant enzymes particularly H₂O₂ specific CAT, in preventing PUFA oxidation of the H₂O₂-stressed RPE cells, and in fact, the DHA depletion via oxidative stress suggest being a predominant contributing factor in pathophysiology of AMD [39].

Little is known about the biological links between oxidized PUFA products (also known as lipid mediators) and the development of AMD. Oxidative stressed RPE cells in this study accumulated a variety of products. These findings provide new insights in the molecular events of lipids in RPE cells following oxidative stress. Past reports indicated the ability of 4-HNE to modify and inactivate retinal proteins by forming adducts that contributes to AMD pathophysiology [40, 41]. Of note, urinary 15-F_{2t}-IsoP was previously

Fig. 2 Levels of antioxidant enzymes of RPE cells (n=3) with or without 600 μM H_2O_2 challenge. Treatment concentrations were 10 μM lutein, 10 μM zeaxanthin and 25 μM DHA. Values are expressed as mean (S.D.). Different lower-case alphabets indicate statistical differences between treatment groups without H_2O_2 challenge; different uppercase capitals indicate statistical differences between treatment groups with H_2O_2 challenge; *** $p < 0.001$ statistical significance with vs without 600 μM H_2O_2 . Zea: zeaxanthin; L+Z: lutein plus zeaxanthin; L+Z+D: lutein plus zeaxanthin plus docosahexaenoic acid



found to be associated with neovascular AMD development [42], whereas elevated 4-HNE was reported in patients with neurodegenerative diseases [43]. Furthermore, recent report showed increased systemic 4-F_{4t}-NeuroP and 10-F_{4t}-NeuroP

in Rett Syndrome patients and other neurological diseases, which suggests them to be potential markers for neurodegeneration including AMD [39]. It is noted 4-F_{4t}-NeuroP levels showed stronger suppression by lutein + zeaxanthin

than DHA alone in the H₂O₂-stressed RPE cells in our observation. This suppression remained when the three were combined. This contradicts to our recent findings [39] where decrease in omega-3 PUFA oxidation, in particular NeuroPs and HDHA enhanced the risk of AMD subjects however, such observation was attributed by low circulating DHA. Although the use of RPE cell line cannot fully represent the human body, our findings suggests sufficient DHA and carotenoids are essential for homeostatic control in oxidative damage of the RPE cells, perhaps through intracellular antioxidant gene expressions.

Taking into account that ROS induces PUFA oxidation and contributes to AMD progression, it of interest to see if supplementation with carotenoids and DHA lessen oxidative damages. Lutein and zeaxanthin are lipid antioxidants and anticipated to protect cellular PUFA from ROS attack. Single treatment of lutein, zeaxanthin, DHA, or lutein + zeaxanthin did not reduce PUFA oxidation or induce changes in cellular gene expressions in this study. Lipoxigenase-mediated DHA oxidation products including NPD1 were increased after treatment with DHA. NPD1 is reported to support homeostatic regulation of retinal epithelial and photoreceptor cell integrity, particularly under oxidative stress [44].

On contrary, combination of lutein + zeaxanthin with DHA possibly protected RPE cells from ROS attack in multiple ways. For instance, it conserved a considerable portion of DHA even under oxidative stress, and simultaneously elevated RvD1, NPD1 and 17-HDHA. These are known to be neuroprotective but insofar its exact role in retinal cells is unknown, except for NPD1. HDHAs are anti-inflammatory and with the pro-resolving RvD, they can possibly prevent degeneration of RPE cells. Nevertheless, when considering how cell culture media also imposes a state of oxidative stress on cells [45], it is uncertain whether the production of these DHA mediators is a cellular event or just a catalysed oxidation caused by ROS in the media.

Expressions of antioxidant related transcriptional factors such as NFE2L2 and PPAR γ were suppressed in H₂O₂-stressed RPE cells when treated with lutein + zeaxanthin or with DHA respectively. In addition, down-regulation of ALOX-5 by lutein + zeaxanthin with DHA and GPx-1 by lutein + zeaxanthin was also observed and coincides with HETEs reduction and GSH elevation respectively. Such observation consistently indicates the combined treatment of lutein + zeaxanthin is protective to retinal cells where a recent report suggested the depletion of GSH induced ferroptosis, autophagy, and more importantly, premature cell senescence in RPE cells [46].

It was anticipated carotenoids and DHA together in the retinal cells would protect each other from oxidation, increase its bioavailability and prevent the formation of toxic compounds like HNE. Our study did not show such kind of

protection and in fact, lutein and zeaxanthin were unable to rescue the low cell viability induced by H₂O₂. DHA treatment was reported to reduce apoptosis and induce a transient increase in ROS in RPE cells, where the subtoxic stress can activate the antioxidant stress response transcription factor NFE2L2/NRF2 (nuclear respiratory factor) that controls the transcriptional encoding of the genes related to endogenous enzymatic and nonenzymatic antioxidants [47]. However, in this study it appears that DHA was unable to induce endogenous antioxidants in RPE cells, thus prevent ROS-driven cell apoptosis.

Although we did not measure both carotenoids in the RPE cells, the uptake may have been limited as they are transported in vivo by lipoproteins (LDL, HDL) [48]. Further, retinal lutein and zeaxanthin may have preferentially formed complexes with ocular proteins. A study reported that the Pi isoforms of glutathione transferase protein (GSTPi) in human macula have greater interaction with zeaxanthin, but not lutein [49]. However, in this study, it was also possible H₂O₂ modified the cellular protein and prevented the formation of the complex. Moreover, membrane-bound lutein is believed to scavenge oxidants by quenching reactive oxygen intermediates through a large number of unbound double bonds in the lutein molecule [50]. The orientation of lutein in the cellular membrane may affect its antioxidant efficiency. Normally, dipolar carotenoids including lutein is located and distributed within the cellular membrane in a vertical manner. Nonetheless, its distribution in retinal cells varies when bound to proteins [51] and transferred into the lipid membrane of the retinal cells. The proximity of transmembrane lutein with polyunsaturated phospholipids allow optimal protection against ROS.

To conclude, H₂O₂ depleted DHA and enhanced lipid oxidation in RPE cells. Seemingly, lutein and zeaxanthin when combined provided better cellular protection from oxidative stress related to non-enzymatic oxidation while DHA displayed its protection by reducing the production of pro-inflammatory mediators of omega-6 PUFA through PPAR γ and ALOX-5 inhibition, and the augmentation of anti-inflammatory mediators of omega-3 PUFA. The combination of lutein, zeaxanthin and DHA did not further benefit in cell protection from oxidative damage and appeared to be mutually exclusive.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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