

Research Article

Clin Nutr Hosp Diet. 2024; 44(S1): 01-13 DOI: 10.12873/0211-6057.44. S1.006

Dietary supplement enriched in antioxidants and omega-3 on human retinal pigment epithelium cells line: Evidence of protective effect against oxidative stress

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Received: 19-Jun-2024, Manuscript No. CNHD-24-139376; **Editor assigned:** 21-Jun-2024, PreQC No. CNHD-24-139376 (PQ); **Reviewed:** 05-Jul-2024, QC No. CNHD-24-139376; **Revised:** 11-Jul-2024, Manuscript No. CNHD-24-139376(R); **Published:** 18-Jul-2024, DOI: 10.12873/0211-6057.44. S1.006.

ABSTRACT

Purpose: Dietary supplement enriched with antioxidants and fish oil had been shown to protect the retina from oxidative stress *in vivo* from light-induced retinal damage as well as Müller cells *in vitro* from H₂O₂ treatment. The objective of the present study was to evaluate the effect of this complex supplement on human retinal pigment epithelium cells line (Arising Retinal Pigment Epithelia-ARPE-19) since RPE (Retinal Pigment Epithelia) is key for nutrients uptake from the choriocapillaris and to the photoreceptors of neural retina.

Methods: We used ARPE-19 cells treated for 7 days with the dietary supplement at 11 or 44 μ M eq. DHA.

Results: Herein, we demonstrated that incubation of ARPE-19 cells for 7 days with the supplement significantly reduced cells death induced by H_2O_2 -oxidative stress from 11 µM and 44 µM equivalent DHA. Interestingly, we showed different mechanism of protection at both concentrations with a direct signal effect at 11 µM and through membrane composition modification at 44 µM. Surprisingly, at 44 µM equivalent DHA, the supplement induces phosphatidylserine externalization, an increase of isoprostanes production and caspase-3/7 activation.

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Conclusion: At this high concentration, pre-conditioning effect seems to take place. Nevertheless, these results raised up the question of the long-term treatment in human.

Keywords: Eye, Fish oil, Oxidative-stress, Apoptosis, Phosphatidylserine, Plasmalogen, Fatty acids/ Oxidation, Isoprostanes, ARPE-19 cells

INTRODUCTION

Age-related Macular Degeneration (AMD) is the leading cause of blindness and visual impairment after 60 years of age in western populations^[1,2]. AMD is characterized by degenerative changes within the macula, which is the central area of the retina responsible for detached vision and colour perception. The early stage of AMD is associated with an accumulation of small focal deposits called drusen under the Retinal Pigment Epithelium (RPE) cells. As the disease progresses, large RPE cell loss in focal areas can occur referred as geographic atrophy which leads to a progressive worsening of central vision. This can be followed by neovascular response arising under the retina disrupting normal retinal anatomy, known as neovascular AMD^[3-7]. Although vascular endothelial growth factor inhibitors injected directly into the vitreous humour of the eye can stabilise vision in neovascular AMD, there is no treatment available for geographic atrophy. In the absence of a cure, research has focused on preventing, notably through nutritional intakes, or slowing the progression of AMD.

Several epidemiological studies (observational or interventional) have reported the benefit of an increased consumption of antioxidant and omega-3 Long Chain Polyunsaturated Fatty Acids (LC-PUFAs), including Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA), in the prevention of AMD. Supplementations with vitamins, trace elements, omega-3 fatty acids and other nutrients based on their antioxidant properties have become standard in clinical care^[8]. As a consequence, supplement to improve vision have exploded on the market. However, little is known on the mechanism involved in the effect of complex supplements. Interestingly, we had previously shown that a dietary supplement enriched with antioxidants and fish oil, commercialized for clinical care of AMD patients, is associated to a protective effect against oxidative stress through modification of glutamate-glutamine cycle in vitro on Müller cells culture^[9] and through changes in retinal fatty acid composition in vivo in a light-induced retinal degeneration model in rat^[10]. In this in vivo experiment, the supplement was given daily by oral administration suggesting that nutrients had to go through RPE cells to reach the retina. RPE cells are involved in the uptake of nutrients from the choriocapillaris and their transfer to the photoreceptors of neural retina and in the elimination of waste from the photoreceptors^[11]. Furthermore, RPE cells are also involved in visual pigment regeneration and photoreceptor outer segment renewal, then participating to maintenance of photoreceptor integrity^[12]. Therefore, RPE cells are essential for photoreceptor cells survival^[13]. In addition, a growing body of clinical and experimental data strongly implicate RPE cells death induced by oxidative-stress in AMD pathogenesis^[14,15].

In order to go further in the mechanisms involved in this complex supplement protection, we have evaluated for the first time the effect of a dietary supplement enriched in antioxidants and omega-3 on RPE cells.

MATERIALS AND METHODS

For our investigation, we used the ARPE-19 cells, a spontaneously transformed human RPE cells that conserve many biological and functional RPE cell properties, generously provided by Dunn et al. (INM, Montpellier, France)^[16]. Cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium (DMEM/ F12) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin and incubated at 37 °C in a 5% CO₂ incubator for use in the subsequent experiments.

ARPE-19 cells were pre-treated with Dietary Supplement

(DS) as shown in Table 1 at several concentrations (0.7 μ M, 1.40 μ M, 2.8 μ M, 5.5 μ M, 11 μ M, 22 μ M, 44 μ M, 88 μ M, 118 μ M, 147 μ M, 177 μ M eq. DHA prepared in DMSO 0.1%) expressed as equivalent of Docosahexaenoic Acid (DHA) concentration, for 7 days. It is important to note that in our previous *in vivo* and *in vitro* experiments, the supplement was used at 11 μ M eq. DHA for 5 to 7 days of treatment corresponding to the initial commercialized concentration of 365 mM^[9,10]. The control group was treated with 0.1% DMSO in growth media.

Table 1. Effect of dietary supplementation on ARPE-19 cells and its composition

Dietary Supplement composition (DS)	Commercial mixture concentration			
Ingredients	(mg/ml)			
Vitamin and trace elements				
Vitamin C	150			
Vitamin E	25			
Zinc (sulfate)	12.5			
Copper (sulfate)	0.83			
Essentials fatty acids				
Fish oil	580			
with 70% omega-3	405.83			
EPA	231.66			
DHA	115.83			
DPA	14.56			
Extract of Tagetes erecta				
Lutein	8.33			
Zeaxanthin	1.66			
Extract of Vitis vinifera				
Resveratrol	0.83			

To induce oxidative stress on ARPE-19 cells, hydrogen peroxide was widely used in the literature^[17-20]. In our experimental design, after 7 days with or without DS, oxidative stress was induced during 2 hours using 600 μ M of Hydrogen Peroxide (H₂O₂) extemporaneously prepared by dissolving 3% H₂O₂ in a serum-free DMEM/F12 medium. This concentration was shown to induce 40%-50% of viability loss (unpublished data).

In our experiments, cell viability and cell death were quantified by MTT 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide assay (Thermofisher) according to manufacturer's instructions and flow cytometry (Cytometer EPICS XL Beckman Coulter, Fullerton, CA) using annexin V-Fluorescein Isothiocyanate (V-FITC) and Propidium Iodide (PI) labelling. Viable cells are annexin V-/IP-, cells in early stage of apoptosis are annexin V+/ IP-and necrotic cells are annexin V+/IP+. The results are expressed as the percentage of total cells. To go further in apoptosis mechanisms, we evaluated caspases-3/7 activation using the ApoTox-Glo Triplex assay (Promega, France) following manufacturer's protocol^[21,22]. Moreover, cell survival assay, the clonogenic test, was performed as previously described by Villalpando and collaborators^[23]. In addition, transcriptomic approach was performed as previously described^[9].

Data analysis was using GraphPad Prism 7.00 and all results are expressed as mean \pm SEM (Standard Error of the Mean). Statistical comparisons among groups were conducted using one-Way ANOVA. If ANOVA was significant, multiple comparisons were done to determine which pairs of mean values were different. Significant differences between groups were assessed with the post hoc Newman-Keuls test. The significance level was set at p=0.05: One symbol for p<0.05, two symbols for p<0.01, three symbols for p<0.001, and four symbols for p<0.0001.

RESULTS AND DISCUSSION

Firstly, we evaluated the toxicity of the DS on ARPE-19 cells. Concentrations between 0.7 µM and 88 µM eq. DHA had no effect on cell viability compared to non-supplemented cells as shown in Figure 1a, green bars whereas DS at 118 μ M, 147 μ M and 177 μ M eq. DHA induced a 41%-53% and 86% decrease in cell viability, respectively. These data show that under our experimental conditions, DS present a dose-dependent toxicity at a concentration over 88 µM eq. DHA as shown in Figure 1a, green bars. Based on this profile, we tested the potential protective effect of Dietary Supplement (DS) against oxidative stress induced by H₂O₂ as shown in Figure 1a, blue bars. In non-supplemented cells (-DS), H₂O₂ at 600 µM induced a 42% decrease (p<0.0001) in cell viability compared to no-H₂O₂ cells as shown in Figure 1a, blue bars as described previously^[17,18]. Overall, we observed a dose dependent protective effect on cell viability of DS from 0.7 to 44 μ M eq. DHA against H₂O₂ as shown in Figure 1a, blue bars. Nevertheless, DS from 0.7 to 2.8 μ M eq. DHA have no significant effect against H₂O₂ as shown in Figure 1a, blue bars, whereas DS from 5.5 μ M to 88 μ M eq. DHA offered more than 50% protection against H₂O₂ as shown in Figure 1b, with full protection at 44 μ M eq. DHA as shown in Figure 1a, blue bars; Figure 1b. Indeed, no more significant difference was observed between 44 μ M eq. DHA treated cells with or without H₂O₂ as shown in Figures 1a and 1b. Over 88 µM eq.DHA, as expected since DS at these concentrations is toxic by itself, DS have no longer protection against H₂O₂-toxicity as shown in Figures 1a and 1b. Therefore, pre-treatment with DS

has a protective effect against oxidative stress.

To better characterize the beneficial effect of the pre-treatment with DS, we evaluated its cellular effect on ARPE-19 cells at several levels without oxidative stress. Based on these results and to better characterise the effect of DS on ARPE-19 cell death or viability, we selected two DS concentrations: One of the first significant protective concentration (11 µM eq. DHA) and the most protective concentration (44 µM eq. DHA). The choice of the lower concentration (11 µM eq. DHA) is reinforced by previous data, since DS at 11 µM eq. DHA had been shown to protect in vitro Müller cells in culture against oxidative stress induced by H2O2[24] and in vivo against light-induced retinal degeneration^[10]. At 11 µM eq. DHA, no evidence of apoptosis (early or late) was observed in flow cytometry as shown in Figure 1c or caspase activation assay as shown in Figure 1d and Table 2. Unexpectedly, cells pre-treated at 44 µM eq. DHA presented both early hallmark of apoptosis^[25,26] characterized by the Phosphatidylserine (PS) externalization as observed via the significant (p<0.0001) 20% increase of Annexin V+/ IP (Propidium Iodide), and late hallmarks of apoptosis as observed by the significant higher (p=0.0118) basal level of caspase-3/7 activity compared to non-treated cells (0 µM eq. DHA) as shown in Figure 1d, full bars. Nevertheless, addition of Staurosporine (S), a pro-apoptotic agent, in medium culture of cell treated with 44 µM eq. DHA cells (S44) increased the caspase activity to a similar level as untreated cells with Staurosporine (S0) as shown in Figure 1d. There is no additive effect of S (Staurosporine) on the upregulated basal activity of caspase at 44 μ M eq. DHA treatment. Consequently, 44 µM eq. DHA treatment impacted only the basal level of caspase-3/7 activity and not cell viability since cells kept their proliferation ability as confirmed by the clonogenic test^[23] as shown in Figure 1e. However, although no evidence of apoptosis (early or late) was observed at 11 µM eq. DHA Staurosporine (11), addition of Staurosporine, induced caspase activation Staurosporine 11 (S11) but at a significant lower level compared to untreated Staurosporine (S0) or 44 µM eq. DHA (S44) treatment. These observations are suggesting that 1/only 11 µM eq. DHA condition induced a protection against apoptosis induction by lowering caspase activation; and 2/7 days of pretreatment with 44 μ M eq. DHA DS increased the apoptotic basal level apoptosis without effect on the cell response to apoptotic stress inducer.

Since retinal pre-conditioning and modification of cell response to oxidative stress could find its origin in the fatty acid composition of membranes^[27], we determine

the fatty acid composition of supplemented ARPE-19 cells. PS externalization might be emphasized by the change in fatty acid composition in supplemented ARPE-19 cells, especially by the 65% increase (p<0.0001) of DHA when cells were incubated with 44 μ m eq. DHA as shown in Table 2. High levels of DHA may lead to general membrane instability resulting in enhanced flip-flop, increasing the likelihood of moving PS to outer leaflet^[28-32]. Moreover, when cells were incubated with 44 µm eq. DHA, the level of Arachidonic Acid (AA, C20:4 n-6) is significantly decreased by 23.6% (p<0.0001). This decrease could be explained by its use to synthesize isoprostanes. Indeed, the Isoprostanes (IsoPs) are a unique series of prostaglandin-like compounds formed via a nonenzymatic mechanism involving the free radical-initiated peroxidation of Arachidonic Acid (AA, C20:4 n-6)^[33]. Among them, the 15-F2t-Isoprostanes (15-F2t-Isop) is considered as a gold standard biomarker of oxidative stress^[27,34-36]. In our experimental condition, 11 µM eq. DHA had no effect on 15-F2t-IsoP production whereas 44 µM eq. DHA increased (p=0.001) 15-F2t-Isop level by 360% compared to untreated cells as shown in Figure 1f with the simultaneous, decrease (p<0.0001) by 23.6% in AA (C20:4 n-6) level. Interestingly, an increase (p<0.0001) by 202% in EPA (20:5n-3) was observed. These concomitant changes could participate to the protective effect by increasing (p<0.0001) the ratio EPA/AA by 106% as shown in Tables 2 and 3. EPA, an n-3 PUFA, is a potent antioxidant and anti-inflammatory agent and regulates the expression of various cytoprotective antioxidant enzymes^[37,38]. In addition, EPA and AA compete for membrane incorporation leading to changes in membrane ratio n-3/n-6 total Polyunsaturated Fatty Acids (PUFA), thereby modulating various signalling pathways^[39]. High n-3/n-6 ratio is key for redox homeostasis in the body^[40,41]. Herein, although PUFA content was not significantly modified in 11 µM eq. DHA treated cells, total PUFA was significantly increased (<0.0001) by 38% in 44 µM eq. DHA treated cells compared to control mainly due to a decreased level of n-6 (-5.6%, <0.0001) and increased level of n-3 (+107.0%, <0.0001) leading to significant increase of n-3/n-6 ratio (+944%, <0.0001). Among n-3, the most striking increased was observed for the precursor of the a-Linolenic Acid (ALA, C18:3 n-3, +40.2%, <0.0001), n-3 Docosapentaenoic Acid (DPA, C22:5n-3, +61.4%, <0.0001) and DHA (C22: 6n-3, +65.2%, <0.0001). Regarding n-6, 44 µM eq. DHA induced an increase of Linoleic Acid (LA, C18:2 n-6, 88.2%, <0.0001), Dihomo-y-Linoleic Acid (DGLA, C20:3 n 6; +46.7%, <0.0001) and a decrease of Docosatetraenoic (DTA, C22:4 n-6; -38.86%, <0.0001) and Arachidonic

Acid (AA, C20:4 n-6; -23.6, <0.0001). Obviously, DS has an important impact on DHA pathway in the membrane. DHA is known to generate docosanoids such as Neuroprotectin D1 (NPD1) which elicits neuroprotective activity in brain ischemia-reperfusion, in oxidative-stressed retinal pigment epithelial cells; And promotes neuronal and glial cell survival^[42,43]. Therefore, the observed increase in DHA precursor might participate to the neuroprotective mechanism by facilitating oxidized-DHA replacement inside the membrane^[44]. In addition, Plasmalogen (Pls), a group of phospholipids with a vinyl-ether bond in the sn-1 position of glycerol^[45] being sensitive markers of oxidative stress^[46,47], are clearly alerted in presence of DS. Indeed, Pls level determined as the relative proportions of Dimethylacetals (DMAs) were reduced (p<0.0001) by 8.5% in cells pre-treated with 44 µM eq. DHA suggesting an increase in oxidative stress which is in accordance with our current data as shown in Figure 1. All together, these data suggest that the cellular effect of 44 μ M eq. DHA, believed to be a "pre-conditioning effect", passed through the modification of cell lipid composition. This observation is in accordance with the literature since it had been shown that the omega-3 fatty acid DHA and its derivatives (i.e. 17-HDHA, NPD1) facilitate cell survival and adaptation to stress in both in vitro and in vivo models of retinal pre-conditioning^[48]. These results are also in accordance with our previous in vivo study showing that a seven-day supplementation was leading to lipid composition modification (especially DHA, EPA and AA) allowing protection to retinal degeneration ^{10]}. Therefore, high concentrations of DHA would act like a "pre-conditioning" via lipid composition changes to ensure protection from a further stress. At the contrary, lower DHA concentrations seem to have a direct effect on cell signalling. Indeed, it appears that the state of the cells before H₂O₂-induced oxidative stress was different depending on the concentration, suggesting two different protective mechanisms thereafter. Indeed, with 11 µM eq. DHA treatment, no variation of lipid composition could be associated with the protection against H₂O₂. In this case, we could suggest that the supplement have a direct cellular signalling effect through the PUFAs it contains as it had been observed in vitro in Müller cells^[9]. Interestingly, transcriptomic investigation at 11 μ M eq. DHA showed few modifications of mRNA expression with 42 genes significantly deregulated as shown in Table 3. In accordance with the protective effect observed, this gene list was significantly enriched in genes involved in stress response as observed in the clustering data (i.e GO:00021666, GO:0009410, GO:0042542) suggesting a direct signalling effect of DS as shown in Figure 2.

Finally, although the dietary supplement tested contains

antioxidants (vitamin E, vitamin C, zinc, resveratrol and



Figure 1a. Cell viability. Green bars represent ARPE-19 cells incubated with DS at different concentrations (from 0.7 μ M to 177 μ M eq. DHA) for 7 days. The results are expressed as the percentage of control condition (0 μ M eq.t DHA=100% of cell viability). Blue bars represent ARPE-19 cells pre-treated with different concentrations of DS and then exposed to 600 μ M H₂O₂ for 2 h. The cell viability was determined MTT assay. The results are expressed as the percentage of control condition (without DS treatment (0 μ M) or H₂O₂ exposure=100% of cell viability). The data are presented as means ± SEM (n=3 independent experiments, each n for each condition represented a triplicate). Significance (\$) compared to 0 μ M éq-DHA. Significance (*) compared to 0 μ M eq. DHA cells treated with H₂O₂

Note: (**1**): 0 μ M eq.t DHA=100%; (**1**): 0 μ M or H₂O₂ exposure=100%; (**1**): Compared to 0 μ M eq. DHA cells treated with H₂O₂



Figure 1b. Representation of the protection against H_2O_2 exposure. Data are obtained from the difference between cell viability of DS-treated cell without and with H_2O_2 treatment (2 h)



Figure 1c. ARPE-19 cells in flow cytometry. Cells were collected and stained with Annexin V-FITC. The results are expressed as the percentage of Annexin V-FITC-stained cells. The data are presented as means \pm SEM (n=3 independent experiments). Significance compared (*) to untreated cells (0 μ M eq. DHA) **Note:** (\Box): 0 Eq. DHA; (\Box): 11 Eq. DHA; (\blacksquare): 44 Eq. DHA



Figure 1d. Caspase-3/7 activation. Ratio Caspase-3/7 activity / Cell viability was evaluated in ARPE-19 cell without or with DS, and without or with stimulation by staurosporine (S, dot bar). The data are presented as means \pm SEM (n=2 independent experiments)

Note: (
): Staurosporine S0; (
): Staurosporine S11; (
): Staurosporine S44

DIETARY SUPPLEMENT ENRICHED IN ANTIOXIDANTS AND OMEGA-3 ON HUMAN RETINAL PIGMENT EPITHELIUM CELLS LINE: EVIDENCE OF PROTECTIVE EFFECT AGAINST OXIDA-TIVE STRESS



Figure 1e. Clonogenic test. Evaluation of ARPE-19 cell proliferation without or with DS was performed by determined the number of clones per mm². The data are presented as means \pm SEM (n=2 independent experiments **Note:** (\Box): Staurosporine S0; (\blacksquare): Staurosporine S1; (\blacksquare): Staurosporine S44



Figure 1f. 15-F2t-IsoP (isoprostanes) level was analysed by gas chromatography. The results are expressed in pg of 15-F2tIsoP in five pooled wells. The data are presented as means \pm SEM (n=2 independent experiments). (H) ARPE-19 cells were treated with two concentrations of DS (11 µM and 44 µM eq. DHA) for 7 days. Fatty acid composition was analysed by gas chromatography. The data are presented as means \pm SEM (n=2 independent experiments). Overall significance (*): One symbol p<0.05; two symbols p<0.01; three symbols p<0.001

Note: (
:): Staurosporine S0; (
): Staurosporine S11; (
): Staurosporine S44

Table 2. ARPE-19 cells were treated with two concentrations of DS (11 μ M and 44 μ M eq. DHA) for 7 days. Fatty acid composition was analysed by gas chromatography. The data are presented as means ± SEM (n=2 independent experiments)

	0 μM Eq. DHA		5.	5.5 μΜ Eq. DHA		44 μM Eq.DHA		5,5 <i>vs.</i> 44 Eq. DHA	
	Mean	SEM	Mean	SEM	Stat <i>vs</i> . 0 µM Eq. DHA	Mean	SEM	Stat <i>vs.</i> 0 µM Eq. DHA	Stat
SFA 14:00 15:00 16:00 17:00 18:00 20:00 22:00	42,77 0,790 0,383 21,328 0,575 18,610 0,575 0,508	0,867 0,063 0,008 0,632 0,082 0,265 0,013 0,017	39,763 0,733 0,320 19,903 0,473 17,338 0,500 0,498	0,167 0,083 0,016 0,337 0,165 0,360 0,030 0,017	NS NS NS NS NS NS NS	37,830 0,555 0,258 18,308 0,530 17,085 0,565 0,530	0,252 0,032 0,005 0,246 0,170 0,104 0,021 0,015	<0,0001 <0,0001 <0,0001 <0,0001 <0,0001 <0,0001 <0,0001	NS NS NS NS NS NS
MUFA 18:1t 16:1n-7 18:1n-7 16:1n-9 18:1n-9 20:1n-9 22:1n-9 24:1n-9	27,253 0,893 1,475 3,835 1,665 18,485 0,275 0,358 0,268	0,230 0,063 0,030 0,065 0,101 0,247 0,009 0,025 0,008	28,160 0,830 1,568 3,800 1,823 19,265 0,325 0,288 0,263	0,131 0,037 0,036 0,046 0,049 0,151 0,009 0,017 0,008	NS NS NS NS NS NS NS NS	24,375 0,893 1,220 3,110 1,273 16,880 0,508 0,263 0,230	0,090 0,103 0,012 0,013 0,021 0,133 0,015 0,010 0,000	0,0039 NS 0,0039 0,0039 0,0039 0,0039 0,0039 0,0039 0,0039	<0,0001 NS <0,0001 <0,0001 <0,0001 <0,0001 <0,0001 <0,0001
PUFA 20:3n-9	23,150 0,273	0,307 0,028	25,245 0,243	0,196 0,011	NS NS	31,943 0,323	0,340 0,146	<0,0001 <0,0001	0,0081 0,0081
omega-6 18:2n-6 20:3n-6 20:4n-6 22:4n-6	14,093 1,838 0,948 10,298 1,010	0,215 0,110 0,016 0,161 0,016	14,813 2,405 1,108 10,450 0,850	0,109 0,030 0,017 0,104 0,018	NS NS NS NS NS	13,298 3,458 1,390 7,833 0,618	0,147 0,045 0,020 0,156 0,009	<0,0001 <0,0001 <0,0001 <0,0001 <0,0001	0,0081 0,0081 0,0081 0,0081 0,0081
omega-3 18:3n-3 20:5n-3 22:5n-3 22:6n-3 omega-3/ omega6	8,853 0,328 2,815 2,075 3,635 0,628	0,097 0,014 0,043 0,029 0,038 0,008	10,190 0,303 3,725 2,145 4,018 4,523	0,100 0,011 0,066 0,048 0,077 0,011	NS NS NS NS NS NS	18,323 0,460 8,508 3,350 6,005 6,558	0,403 0,016 0,196 0,086 0,155 0,080	<0,0001 <0,0001 <0,0001 <0,0001 <0,0001 <0,0001	0,0081 0,0081 0,0081 0,0081 0,0081 0,0081
Total DMAs dma16:0 dma18:0 dma18:1n-7 dma18:1n-9	6,395 1,870 2,570 1,190 0,765	0,108 0,024 0,065 0,017 0,016	6,833 1,978 2,985 1,215 0,655	0,015 0,032 0,027 0,005 0,010	NS NS NS NS NS	5,850 1,770 2,845 815 420	0,127 0,036 0,076 0,010 0,009	0,0499 0,0499 0,0499 0,0499 0,0499 0,0499	<0,0001 <0,0001 <0,0001 <0,0001 <0,0001

Table 3. List of gene with mRNA expression deregulated by the DS at 11 μ m eq. DHA

ID	Gene symbol	Description	Fold change	P-val	FDR P-val
TC0500012459.hg.1	PDGFRB	Platelet-derived growth factor receptor, beta polypeptide	-1,87	2,14E-06	3,00E-04
TC1700007791.hg.1	RARA	Retinoid acid receptor, alpha	-1,78	2,13E-05	1,20E-03
TC1200007819.hg.1	CDK2	Cyclin-dependent kinase 2	-1,75	2,64E-06	3,00E-04
TC2200007906.hg.1	BID	BH3 interacting domain death agonist	-1,72	2,87E-05	1,50E-03
TC1800008650.hg.1	SMAD7	SMAD family member 7	-1,71	2,02E-05	1,20E-03
TC2000006642.hg.1	BMP2	Bone morphogenetic protein 2	-1,71	1,00E-04	4,10E-03
TC0100011621.hg.1	TGFB2; TGFB2-OT1	Transforming growth factor beta 2; TGFB2 overlapping transcript 1	-1,7	9,85E-07	2,00E-04
TC0100010284.hg.1	PEA15	Phosphoprotein enriched in astrocytes 15	-1,46	6,18E-05	2,40E-03
TC1500009082.hg.1	BMF	Bcl2 modifying factor	-1,45	4,00E-04	7,60E-03
TC1200009876.hg.1	CD69	CD69 molecule	-1,43	3,65E-02	1,45E-01
TC0100008664.hg.1	GADD45A	Growth arrest and DNA-damage-inducible, alpha	-1,39	2,00E-04	5,00E-03
TC1100006815.hg.1	WEE1	WEE1 G2 checkpoint kinase	-1,39	4,30E-03	3,74E-02
TC2200007150.hg.1	TIMP3	TIMP metallopeptidase inhibitor 3	-1,37	8,00E-04	1,25E-02
TC1200006888.hg.1	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-1,37	2,70E-03	2,76E-02
TC0200016682.hg.1	PPP3R1	Protein phosphatase 3, regulatory subunit B, alpha	-1,37	2,00E-03	2,26E-02
TC1700011919.hg.1	CEP295NL; TIMP2	CEP295 N-terminal like; TIMP metallopepti- dase inhibitor 2	-1,36	7,00E-04	1,11E-02
TC0800012001.hg.1	PTK2	Protein tyrosine kinase 2	-1,35	1,40E-03	1,81E-02
TC1600006652.hg.1	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	-1,33	4,30E-03	3,76E-02
TC1600009202.hg.1	CREBBP	CREB binding protein	-1,33	4,50E-03	3,86E-02
TC1200006909.hg.1	EMP1	Epithelial membrane protein 1	-1,33	8,30E-03	5,72E-02
TC0500007831.hg.1	F2R	Coagulation factor II (thrombin) receptor	-1,3	1,50E-03	1,93E-02
TC0500009706.hg.1	SQSTM1	Sequestosome 1	1,31	7,00E-04	1,15E-02
TC1100012708.hg.1	FEZ1	Fasciculation and elongation protein zeta 1	1,31	4,10E-03	3,60E-02
TC1000008388.hg.1	FAS	Fas cell surface death receptor	1,31	2,24E-02	1,07E-01
TC0300013949.hg.1	SATB1	SATB homeobox 1	1,34	2,00E-04	5,10E-03
TC1100007273.hg.1	CD44	CD44 molecule (Indian blood group)	1,37	1,00E-04	3,80E-03
TC1600011398.hg.1	MMP2	Matrix metallopeptidase 2	1,37	3,30E-03	3,13E-02
TC0200013261.hg.1	RETSAT	Retinol saturase (all-trans-retinol 13,14-reduc- tase)	1,37	6,10E-03	4,71E-02
TC0900007576.hg.1	ANXA1	Annexin A1	1,38	1,23E-05	9,00E-04
TC1200012643.hg.1	ERBB3	Erb-b2 receptor tyrosine kinase 3	1,38	2,46E-02	1,13E-01
TC1200010968.hg.1	DDIT3	DNA-damage-inducible transcript 3	1,39	1,70E-03	2,05E-02
TC0100013818.hg.1	PPT1	Palmitoyl-protein thioesterase 1	1,39	1,70E-03	2,04E-02
TC1400007227.hg.1	LGALS3	Lectin, galactoside-binding, soluble, 3	1,44	4,00E-04	7,60E-03
TC1800007471.hg.1	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	1,45	7,00E-03	5,11E-02

SOD2	Superoxide dismutase 2, mitochondrial	1,46	8,91E-05	3,00E-03
СТН	Cystathionine gamma-lyase	1,51	9,46E-05	3,20E-03
CAV1	Caveolin 1	1,51	3,70E-06	4,00E-04
SAT1	Spermidine/spermine N1-acetyltransferase 1	1,55	1,00E-03	1,42E-02
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	1,55	1,50E-03	1,86E-02
IGFBP6	Insulin like growth factor binding protein 6	2,26	6,32E-08	3,65E-05
IL6	Interleukin 6	2,81	3,44E-09	6,22E-06
HMOX1	Heme oxygenase 1	3,8	2,29E-09	4,50E-06
	SOD2 CTH CAV1 SAT1 HGF IGFBP6 IL6 HMOX1	SOD2Superoxide dismutase 2, mitochondrialCTHCystathionine gamma-lyaseCAV1Caveolin 1SAT1Spermidine/spermine N1-acetyltransferase 1HGFHepatocyte growth factor (hepapoietin A; scatter factor)IGFBP6Insulin like growth factor binding protein 6IL6Interleukin 6HMOX1Heme oxygenase 1	SOD2Superoxide dismutase 2, mitochondrial1,46CTHCystathionine gamma-lyase1,51CAV1Caveolin 11,51SAT1Spermidine/spermine N1-acetyltransferase 11,55HGFHepatocyte growth factor (hepapoietin A; scatter factor)1,55IGFBP6Insulin like growth factor binding protein 62,26IL6Interleukin 62,81HMOX1Heme oxygenase 13,8	SOD2Superoxide dismutase 2, mitochondrial1,468,91E-05CTHCystathionine gamma-lyase1,519,46E-05CAV1Caveolin 11,513,70E-06SAT1Spermidine/spermine N1-acetyltransferase 11,551,00E-03HGFHepatocyte growth factor (hepapoietin A; scatter factor)1,551,50E-03IGFBP6Insulin like growth factor binding protein 62,266,32E-08IL6Interleukin 62,813,44E-09HMOX1Heme oxygenase 13,82,29E-09



Figure 2. Gene Ontology (GO) terms significantly enriched among differentially expressed genes in ARPE-19 cell treated with DS. Red bars indicate the fold enrichment of the Gene Ontology (GO) and blue bars indicate the gene count. Four enriched clusters respectively enriched with genes involved in response to stress metabolism were detected **Note:** (**–**): Gene count; (**–**): Fold enrichment

carotenoids (lutein and zeaxanthin)) beside fish oil, we think that the protection is mainly due to the fatty acids. Indeed, it had been shown that vitamin C pre-treatment of ARPE-19 cells in vitro reduced transcriptional activation of AP-1, a nuclear transcription factor involved in oxidative stress response, but at a concentration of 100 and 200 μ M while we evaluated 13 μ M of vitamin C at 11 μ M eq. DHA and 105 µM of vitamin C at 44 µm eq. DHA of supplement. Vitamin E acts by both quenching oxy-radicals and recycling the a-tocopheroxyl radical resulting from vitamin E scavenging an oxy-radical^[49]. Resveratrol has to be used between 25 µM and 100 µm to have an antioxidant protective effect against H₂O₂-induced cell death^[50,51] whereas in the supplement the concentration reaches only 0.45 µm for at 44 µm eq. DHA of supplement. However, even at those low and separately ineffective concentrations, we can't exclude synergic effect of these compounds which may confer additional protective effects by attenuating the propagation of oxidative stress^[52-55].

CONCLUSION

We have shown that a complex ocular dietary supple-

ment containing fish oil, antioxidants and resveratrol is associated in changes in fatty acid composition in ARPE-19 cells. Although it protected the cells from H_2O_2 -induced oxidative stress at 11 and 44 µM eq.DHA, the toxic effect at high concentration raised the question of the use of such a supplement over a long period of time in humans. Studies on fatty acid, antioxidants or any others compounds used in supplementation should be conducted over a long period to evaluate chronic toxicity in complement to acute toxicity.

ACKNOWLEDGMENT

None

FUNDING

The research was supported by CHU of Clermont-Ferrand, University of Clermont-Ferrand, CHR of Orléans, CNRS of Orléans, University of Orléans. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURE STATEMENT

None

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

AUTHOR'S CONTRIBUTIONS

Conceptualization: IRC; Data curation: KRBO, AM, AA, FL, SG, NA, OP, IRC; Formal analysis: KRBO, AM; Funding acquisition: IRC; Investigation: KRBO, AM; Methodology: AM, IRC; Project administration: IRC; Resources: IRC; Software: None; Supervision: IRC, OP; Validation: IRC, OP; Visualization: None; Roles/Writing-original draft: OP, IRC, AM; Writing-review and editing: KRBO, AM, AA, FL, SG, NA, OP, IRC.

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