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Article

Comparison of Antioxidant Properties of Dehydrolutein with Lutein and Zeaxanthin, and their Effects on Retinal Pigment Epithelial Cells

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Abstract: Dehydrolutein accumulates in substantial concentrations in the retina. The aim of this study was to compare antioxidant properties of dehydrolutein with other retinal carotenoids, lutein and zeaxanthin, and their effects on ARPE-19 cells. The time-resolved detection of characteristic singlet oxygen phosphorescence was used to compare the singlet oxygen quenching rate constants of dehydrolutein, lutein, and zeaxanthin. The effects of these carotenoids on photosensitized oxidation were tested in liposomes, where photooxidation was induced by light in the presence of photosensitizers, and monitored by oximetry. To compare the uptake of dehydrolutein, lutein, and zeaxanthin, ARPE-19 cells were incubated with carotenoids for up to 19 days, and carotenoid contents were determined by spectrophotometry in cell extracts. To investigate the effects of carotenoids on phototocytotoxicity, cells were exposed to light in the presence of rose bengal or all-*trans*-retinal. The results demonstrate that the rate constants for singlet oxygen quenching are 0.77x1010, 0.55x1010, and 1.23x1010 M-1s-1 for dehydrolutein, lutein and zeaxanthin, respectively. Overall, dehydrolutein is similar to lutein or zeaxanthin in protection of lipids against photosensitized oxidation. ARPE-19 cells accumulate substantial amounts of both zeaxanthin and lutein but no detectable amounts of dehydrolutein. Cells pre-incubated with carotenoids are equally susceptible to photosensitized damage as cells without carotenoids. Carotenoids provided to cells together with the extracellular photosensitizers offer partial protection against photodamage. In conclusion, the antioxidant properties of dehydrolutein are similar to lutein and zeaxanthin. The mechanism responsible for its lack of accumulation in ARPE-19 cells deserves further investigation.

Keywords: carotenoid; lutein; zexanthin; dehydrolutein; retina; retinal pigment epithelium; singlet oxygen; photosensitized oxidation; age-related macular degeneration.

1. Introduction

3′-Dehydrolutein, ((3R,6′R)-3-hydroxy- β,ε-caroten-3′-one also sometimes referred to as 3′-oxolutein or 3′-ketolutein) has been identified in substantial concentrations in human and monkey liver, blood serum and ocular tissues [1-10]. It has been suggested that dehydrolutein is a product of oxidative transformation of dietary carotenoids, lutein (β, ε-carotene-3,3′-diol) and zeaxanthin (β, β -carotene-3,3′-diol). Out of the approximately 40 carotenoids present in a typical human diet, lutein and zeaxanthin are the only ca-

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rotenoids of dietary origin that accumulate in the retina [11-15]. Antioxidant properties of lutein and zeaxanthin have been widely investigated, and it has been well documented that they are very efficient quenchers of excited electronic states of photosensitizers and singlet oxygen, and can act as scavengers of free radicals [15-17].

The ability of lutein, zeaxanthin and their derivatives to quench electronically excited states is of physiological importance because these carotenoids accumulate in the areas of human body, such as the skin and eye, where, due to exposure to ultraviolet or visible light in the presence of photosensitisers, generation of an excited electronic states of photosensitizers and singlet oxygen can occur [18-22]. Moreover, in the macular part of the retina, where lutein and zeaxanthin accumulate in particularly high concentrations, they can act as optical filters absorbing incoming blue light, thereby preventing blue light from reaching the parts of photoreceptive neurons and retinal pigment epithelium (RPE), where visual pigments are present and potent photosensitizers can accumulate [14,23-31]. Therefore, the macular carotenoids are credited with improving visual functions and protecting photosensitive parts of the retina from blue-light-induced oxidative damage. Altogether, the antioxidant and blue-light filtering properties of lutein and zeaxanthin are believed to play an important role in protection of the retina against oxidative stress, and therefore in protection from the development and progression of age-related macular degeneration, the primary cause of blindness in the elderly in developed countries [15,16,32,33].

It has been determined that about 15 to 25% of retinal carotenoids are present in the photoreceptor outer segments, and also, in smaller concentrations, in the RPE [34,35]. The photoreceptor outer segments are the parts of photoreceptive neurons where visual pigments are present, and where, following absorption of light, all-*trans*-retinal can be released from photoactivated visual pigments and accumulate in high concentration [21,34-36]. All-*trans*-retinal is a potent photosensitizer, with an absorption spectrum that extends into the visible range. In the presence of oxygen, 30% of photons absorbed by all-*trans*-retinal can be used for photosensitized generation of singlet oxygen [37]. Therefore, the ability of lutein, zeaxanthin and their derivatives to quench singlet oxygen deserves particular attention. Lutein and zeaxanthin each have the ability to deactivate singlet oxygen and have bimolecular rate constants approaching the diffusion-controlled limits. The deactivation of the singlet oxygen excited states by carotenoids proceeds mainly via a safe route of energy transfer from the excited state of molecular oxygen to the carotenoid molecule, followed by thermal relaxation of the carotenoid triplet state to the ground state releasing excess energy in the form of heat [38].

Dehydrolutein is present in the human macula at substantial concentrations and increases with age [1,3-5,8,10,39]. Post-mortem quantification of dehydrolutein in 4 mm diameter trephine retinal biopsies centred on the maculae from cadavers of <48 years of age (average age of 32+/-8, n=35) revealed 0.6+/-0.5 ng (0.048 ± 0.040 ng/mm^2). In people > 48 years of age (average age of 68+/-7, n=38), it reaches the concentration of 1.3+/-1.8 ng in an identical retinal area (0.10±0.14 ng/mm^2), with the highest total value detected being about 12.5 ng [1,3,39]. Yet, the antioxidant properties of dehydrolutein and its effects on cultured RPE cells have not been investigated. Therefore, the aim of this study was to compare the abilities of dehydrolutein, lutein, and zeaxanthin to i) quench singlet oxygen, ii) protect from photosensitized oxidation of lipids, and iii) compare their effects on RPE cells in the absence and presence of photosensitizers and light.

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2. Materials and Methods

2.1. General chemicals and reagents

Cholesterol, egg yolk phosphatidylcholine (EYPC), all-*trans*-retinal, 93-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), butylated hydroxytoluene (BHT), Chelex 100, rose bengal (95%) were obtained from Sigma-Aldrich Chemical Co. Dehydrolutein was synthesized as described previously [40]. Lutein (95% pure with 5% of zeaxanthin) and zeaxanthin were a generous gift from DSM Nutritional Products AG (Basel, Switzerland). The spin probe, 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrroline-1-yloxy (mHCTPO) was a generous gift from Professor Howard Halpern, University of Chicago, IL, USA. Unless stated otherwise, all procedures involving photosensitizers and/or carotenoids were performed under dim light.

2.2. Determination of singlet oxygen quenching by dehydrolutein, lutein and zeaxanthin

To determine the rate of singlet oxygen quenching, singlet oxygen was generated by photoexcitation of all-*trans*-retinal solution in benzene with a 5 ns laser pulse of the third harmonic of a Q-switched Nd:YAG laser (Continuum Surelite II-10, Photonic Solutions Plc., Edinburgh, UK), and monitored by detection of its infrared emission at 1270 nm by liquid-nitrogen-cooled germanium diode (Applied Detectors Co., Fresno, CA), connected to an Agilent 54830B digitizer (Agilent Technologies UK Ltd.) and analyzed by a RISC workstation operating the LKS.60 nanosecond time-resolved laser photolysis spectrometer (Applied Photophysics, Leatherhead, UK) [19,37,41]. The absorbance of all-*trans*-retinal in a 1 cm square cuvette was about 0.17 at the excitation wavelength of 355 nm for all the solutions. The rate constant for the quenching of singlet oxygen by carotenoids was determined by quantifying the rate of decay of its infrared phosphorescence in the presence of different concentrations of carotenoids [18].

2.3. Preparation of lipid vesicles (liposomes)

Multi-lamellar liposomes were prepared either from EYPC or a mixture of EYPC, DMPC and cholesterol in the presence and absence of all-*trans*-retinal or carotenoids [42,43]. The compounds were dissolved in chloroform in a round-bottom flask, and then the chloroform was evaporated under a stream of argon to form a lipid film. To ensure a complete removal of chloroform, the films were dried under vacuum for at least 1 h. Then the lipid films were hydrated in phosphate buffered saline (PBS). PBS was prepared in deionized glass distilled water and treated with chelating resin, Chelex 100 in order to remove any contaminating metal ions that may catalyse the decomposition of peroxides. For experiments with all-*trans*-retinal, carotenoids were added to the suspension of liposomes from their stock solutions in DMSO, giving the final concentration of 1% DMSO and up to 0.04 mM carotenoids.

2.4. Comparison of the effects of dehydrolutein, lutein and zeaxanthin on photosensitized oxidation of lipids

To determine the effect of carotenoids on photosensitized oxidation mediated by rose bengal or all-*trans*-retinal, electron spin resonance (ESR) oximetry was used as described previously [42,44,45]. In short, oxygen concentrations were monitored by spectral characteristics of 0.1 mM mHCTPO employed as a nitroxide spin probe. The suspensions of lipid vesicles in the presence of rose bengal or all-*trans*-retinal, selected concentrations of carotenoids, and 0.1 mM mHCTPO were irradiated in situ in a flat quartz cell (Wilmad Glass. Co., NJ, USA), in a resonant cavity of the ESR spectrometer, at ambient tempera-

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ture using a 150 W xenon arc lamp (Oriel Corporation, Stratford, CT, USA; 06497 Model 60100) equipped with a combination of lenses and filters (a 5 g/l copper sulphate solution with 10 cm optical pathlength, a glass cut-off filters and an interference filters). For experiments with all-*trans*-retinal, a cut-off filter absorbing light < 390 nm, and an interference filter transmitting light of 404±6 nm were used, and the irradiance inside the resonant cavity, measured with a photodiode PD Irradiance Meter (Hamamatsu, Photonics, K.K., Hamamatsu City, Japan), was 8.1 mW/cm^2. For experiments with rose bengal as a photosensitizer, a cut-off filter absorbing light < 520 nm, and an interference filter 542±4 nm were used, and the irradiance of the sample was 14.0 mW/cm^2. The temperature variation was minimized by continuous flow of gaseous nitrogen through the resonant cavity. ESR measurements were performed using Bruker ESP 300E spectrometer operating at the X band (Bruker, Rheinstetten, Germany). The instrument settings were: microwave power of 1 mW, modulation amplitude of 0.1 G, sweep width of 3.0 G, time constant of 10.24 ms, and conversion time of 20.48 ms.

2.5. Cell culture and feeding with carotenoids

ARPE-19 cells, a spontaneously immortalized and well-characterized human retinal pigment epithelial cell line derived from a 19-year-old male donor, were purchased from the Americam Type Culture Collection (ATCC, Manassas, VA) [46]. ARPE-19 cells were routinely passaged by dissociation in 0.05 % (w/v) trypsin, maintained at 37^{0}C in a humidified incubator filled with 5% CO2 in air, and fed every 2 to 3 days with minimal essential medium (MEM, Sigma Chemical Co., St Louis, MO, USA), containing 10% heat-inactivated foetal calf serum (FCS), L-glutamine and penicillin-streptomycin (Sigma-Aldrich). All experiments were performed on confluent cell monolayers, passage numbers between 25 and 29, seeded either in 75 or 25 cm^2 flasks, or in 24- or 12-well plates [19,47].

2.6. Administration of carotenoids to cultures of RPE cells

Stock solutions of carotenoids were prepared under argon in DMSO under dim light. To facilitate binding of carotenoids to serum lipoproteins and albumin, the carotenoid solution was added to FCS and incubated for 1 h at 37°C with mixing. Then the carotenoid-enriched FCS was added to the culture medium giving a final concentration of 2 μM carotenoids, 0.2% DMSO and 10% FCS. Confluent monolayers of cells, usually 10 days after seeding, were fed with freshly prepared carotenoid-enriched culture medium 3 times a week for up to 19 days.

Alternatively, carotenoids were injected directly into Dulbecco's PBS with calcium and magnesium (DPBS), and incubated with cultured cells for 60 minutes during exposure to photosensitizers and light. After the incubation, cells were washed with DPBS, and then DPBS was replaced with the culture medium for further culture or the reductive activity assay.

2.7. Evaluation of carotenoid content in cells

To determine carotenoid uptake by ARPE-19 cells, the cells were trypsinized, 40 uM aliquots were taken for cell counting, and the remaining cell suspensions were mixed at 5:8 ratio with chloroform/methanol (2:1, v/v) containing 1mM BHT to prevent oxidation [48]. The mixture as vortexed, followed by centrifugation to separate the phases. The bottom chloroform-rich phase was removed, replaced by chloroform phase obtained by centrifugation of a mixture of PBS with chloroform and methanol, and the extraction was repeated. Combined chloroform phases were dried under nitrogen and resolubilized in acetone or chloroform prior to spectrophotometric detection of the accumulated carote-

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noids. The absorption spectra of solubilized extracts were measured in a 0.8 ml cuvette with a 1 cm optical pathlength.

2.8. Evaluation of cell viability

The morphology of the RPE monolayer was routinely observed down the inverted microscope before the media changes and after experimental treatments. Cell viability was quantified by measurements of their reductive activity by the MTT assay, and after trypsinization by cell counts [19,47,48]. Cells were counted in a Bürker chamber. For the MTT assay, cells were washed with DPBS, and then incubated for 60 min in serum-free MEM containing 0.5 mg/ml MTT. Then the cells were washed with DPBS and solubilised with isopropanol acidified with 0.5% HCl. Optical density of the solubilised formazan was read at 570 nm. The reductive activities of cells exposed to carotenoids in the absence of photosensitizers are expressed relative to DMSO-treated cells from the same plate and given as a percentage. The reductive activities of cells exposed to DMSO/carotenoids in the presence of photosensitizers are expressed as a ratios to cells exposed to DMSO/carotenoids in the absence of photosensitizers in the same plate, and given as a percentage.

2.9. Exposure of RPE cells to light and photosensitizers

To test whether carotenoids can affect phototoxicity, ARPE-19 cells were washed from the culture medium with DPBS, and the medium was replaced with DPBS containing 0.5 μM rose bengal or liposomes containing 0.5 mM all-*trans*-retinal and 2.8 mg/ml EYPC. The 24-well plates with cells were placed on the top of a glass sheet above the set of fluorescent tubes, and cells were irradiated at 24-26^{0}C with white fluorescent light for 60 minutes. The irradiance, measured with IL2000 Spectrotube spectroradiometer (International Light Inc., Newbury, MA, USA), was 0.46 mW/cm^2. After selected irradiation times, the cells were washed with PBS and fed with culture medium. MTT assay was performed immediately after exposure, or 24 hours post-exposure.

2.10. Statistical analysis

Unless stated otherwise, results are expressed as means ± SDs from at least 3 independent experiments. Statistical analyses were performed using SigmaPlot14 and one-way ANOVA followed by all pairwise multiple comparison procedures using Hol-Sidak method, where $P<0.05$ were considered statistically significant.

3. Results

3.1. Singlet oxygen quenching

To compare the ability of dehydrolutein to that of lutein or zeaxanthin to quench singlet oxygen, we used a 5 ns laser pulse excitation of all-*trans*-retinal to generate singlet oxygen and monitored the rate of its decay in the absence and presence of increasing concentrations of dehydrolutein, lutein or zeaxanthin (Fig. 1A). For all three carotenoids, the rates of singlet oxygen decay increased in the linear way with increasing carotenoid concentration (Fig. 1B). This allowed the calculation of the singlet oxygen quenching rate constants for each carotenoid. Our results demonstrated that lutein and zeaxanthin exhibited quenching rate constants of $(0.55\pm0.02)\times10^{10}$ and $(1.23\pm0.02)\times10^{10}$ $M^{-1}s^{-1}$, respectively, which are similar to the values reported in literature. Previously reported values for singlet oxygen quenching in benzene are 1.2×10^{10} $M^{-1}s^{-1}$ and 0.66×10^{10} $M^{-1}s^{-1}$ for zeaxanthin and lutein, respectively [38]. The rate constant of singlet oxygen quenching by dehydrolutein, $(0.77\pm0.02)\times10^{10}$ $M^{-1}s^{-1}$ was intermediate between the values for lutein and zeaxanthin, demonstrating that dehydrolutein is also an effective singlet oxygen quencher.

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This is important because zeaxanthin and lutein in the retina appear to be bound with high selectivity and specificity to glutathione S-transferase Pi isoform (GSTP1) and steroidogenic acute regulatory domain protein 3 (StARD3) proteins, respectively [8,49-53], which would limit their diffusion and thereby efficiency as singlet oxygen quenchers [54]. Lacking a specific bind protein, dehydrolutein would be more likely to be present in the lipid membranes as a free carotenoid, and thereby may be more physiologically effective as a singlet oxygen quencher protecting components of the lipid membrane from photosensitised oxidation.

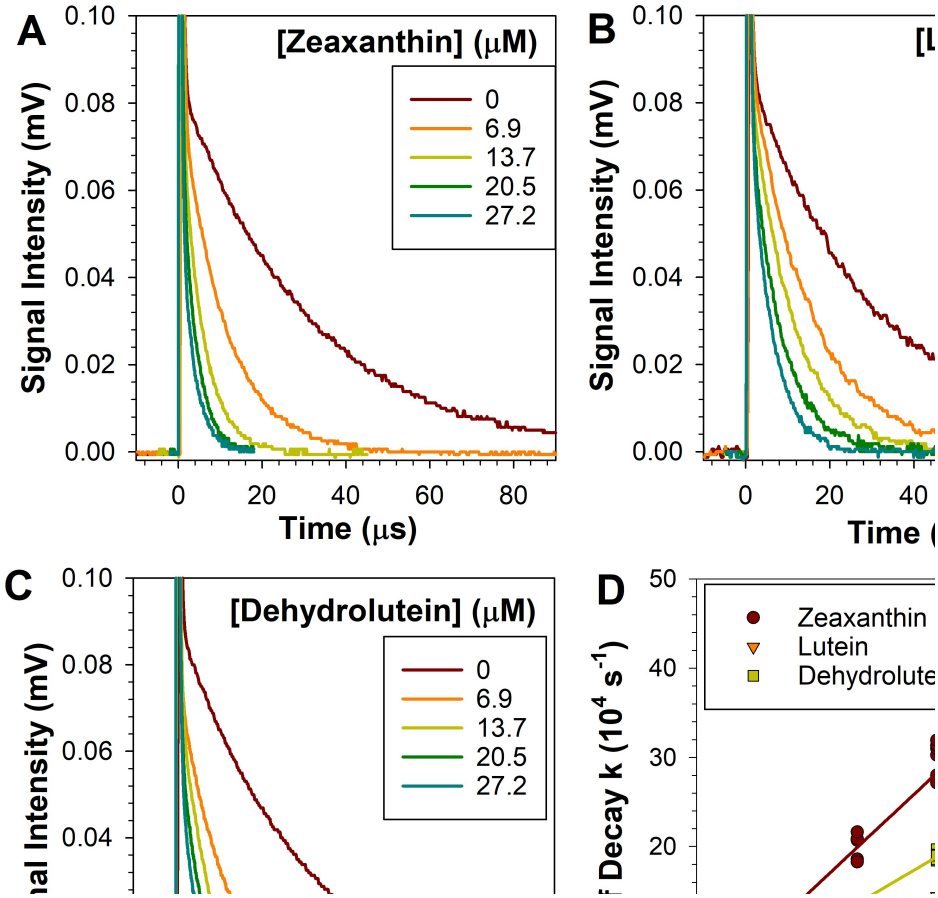

Figure 1. Dehydrolutein quenches singlet oxygen with a similar efficiency to that of lutein or zeaxanthin. A-C: Representative kinetics of formation and decay of singlet oxygen after a 5 ns laser pulse photoexcitation of all-*trans*-retinal in the absence and presence of dehydrolutein, lutein, and zeaxanthin at indicated concentrations. Time 0 indicates when the sample was exposed to the laser flash. The decays were fitted to exponential decay curves to obtain fitted parameters corresponding to the rates of decay. D: Rates of singlet oxygen decay plotted as a function of carotenoid concentration. The quenching rate constants were determined by fitting straight lines to obtain the fitted slopes, which correspond to the rates of quenching.

1.1. Effects of dehydrolutein, lutein and zeaxanthin on photosensitized oxidation

We have shown previously that zeaxanthin can inhibit lipid oxidation when incorporated into lipid vesicles (liposomes) and exposed to rose bengal and green light [42]. Here we used a similar model system to compare effects of dehydrolutein, lutein, and zeaxanthin on rose-bengal-mediated oxidation (Fig. 2). Liposomes containing

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unsaturated lipids in the absence of presence of carotenoids were exposed to rose bengal and light to induce photooxidation which was monitored by measurement of oxygen consumption. At 10 μM concentrations, all three carotenoids significantly decreased the rate of oxygen consumption. Zeaxanthin showed the greatest effectiveness in slowing down the initial rate of oxidation by 47%, followed closely by dehydrolutein which decreased the rate by 45%, but that 2% difference was not statistically significant. Lutein at 10 μM concentration was the least effective of the three carotenoids producing a decrease in the rate of oxidation by 32%, and its effect was statistically different fromthat of both dehydrolutein and zeaxanthin (p=0.005 and 0.002, respectively). Increasing concentrations of carotenoids to 20 μM brought about greater decreases in the rates of oxidation of 60%, 67% and 69% for lutein, dehydrolutein, and zeaxanthin, respectively, with no statistically significant differences between any of the carotenoids. At 40 μM concentrations, the carotenoids decreased the initial rate of oxidation by 97-98%, and all three carotenoids were similarly effective.

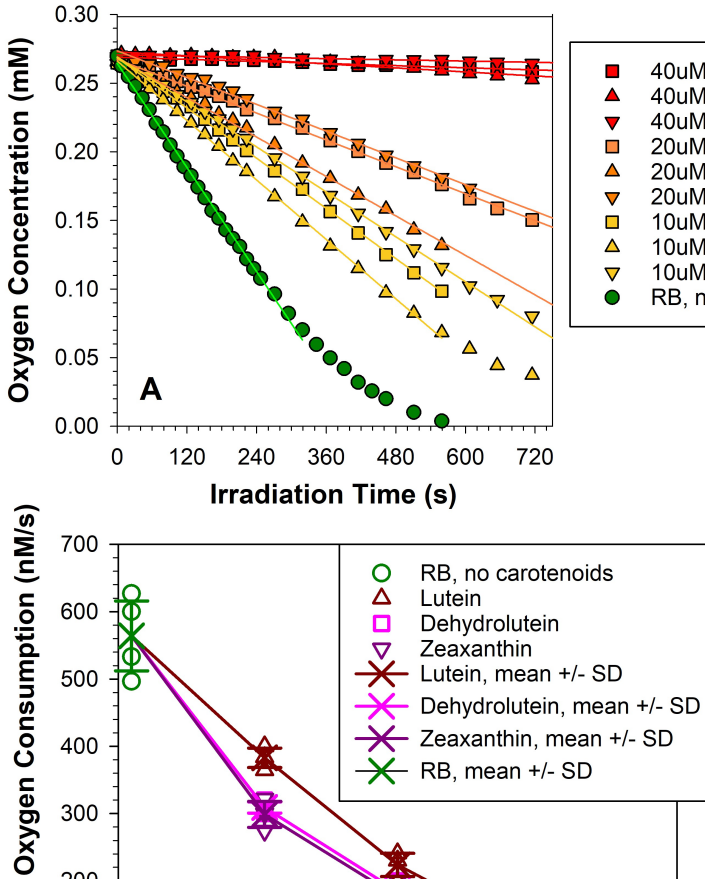

Figure 2. Dehydrolutein inhibits rose-bengal-mediated photooxidation with similar efficiency to lutein and zeaxanthin. A) Representative kinetics of oxygen consumption during exposure to rose bengal and green light of liposomes in the absence and presence of carotenoids at indicated concentrations. Liposomes, consisting of 1 mg/ml egg yolk phosphatidylcholine (EYPC), 2.6 mM cholesterol, 11.8 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and indicated concentration of carotenoids, were

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exposed to green light (542 ± 4 nm; 14.0 mW/cm²) in the presence of 16 μM rose bengal (RB). The samples included 0.1 mM , 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrroline-1-yloxy (mHCTPO) used as a spin probe. The initial linear portions of the kinetics were fitted to straight lines, and the slopes of these lines gave the initial rates of oxygen consumption. B) The initial rates of oxygen consumption in the absence and presence of indicated carotenoids as a function of carotenoid concentration. Statistically significant differences between the initial rates of oxygen consumption in the presence of carotenoids used at the same concentration were only for 10 μM lutein, which was significantly faster than for 10 μM dehydrolutein or zeaxanthin (p=0.005 and 0.002, respectively).

Rose Bengal is a water-soluble photosensitizer which associates with the lipid membrane, but does not penetrate through it [47]. Its photochemical properties are well characterized and its effects on cultured ARPE-19 cells have been previously tested [47]. It is used in ophthalmic practice for diagnostic purposes on the surface of the eye and as a potential agent used for corneal photocrosslinking, it does not penetrate inside the eye and has no physiological effects on the retina [55]. Here we compare the effect of the three carotenoids on oxidation mediated by photosensitization of oxygen by all-*trans*-retinal, a form of vitamin A that can reach light levels in the photosensitive structures of the retina (Fig. 3). As mentioned already, all-*trans*-retinal is a lipophilic photosensitizer that absorbs ultraviolet, violet and blue light and can accumulate in the retina in photoreceptor outer segments during the normal physiological mechanism of retinal during light detection [19,21,36,56,57]. Under the experimental conditions used, in the absence of carotenoids, all-*trans*-retinal and blue light induced 6.5-fold faster oxygen consumption than that induced by rose bengal and green light (Fig. 2, 3). All three carotenoids exhibited effective photoprotection against photoxidation induced by all-*trans*-retinal and blue light at 10 μM concentration ($p<0.001$, in all three cases), decreasing the rate of oxygen consumption by 30, 34, and 45%, respectively for lutein, dehydrolutein, or zeaxanthin. There was no significant difference between samples containing 10 μM lutein and dehydrolutein. Zeaxanthin was more effective that either lutein or dehydroluten ($p<0.001$, in both cases) (Fig. 3). At 20 μM concentration, lutein decreased the rate of oxygen consumption by 68% and was less effective than dehydrolutein or zeaxanthin ($p<0.001$, in both cases), which decreased the rates by 72 and 75%, respectively. There was no significant difference in protection offered by either dehydrolutein or zeaxanthin at this concentration. At 40 μM concentration, the carotenoids decreased the rates of oxygen consumption from 88 to 92% and there were no significant differences between any of the carotenoids.

Altogether, the differences between these carotenoids abilities to slow down photooxidation could be seen only at concentration of 10 μM. Dehydrolutein was more effective than lutein, and similar to zeaxanthin in its protection of lipids from photosensitized oxidation mediated by rose bengal. Protection against all-trans-retinal-induced photooxidation by dehydrolutein or zeaxanthin was equally effective, while lutein was slightly less effective. At 20 and 40 μM concentration all three carotenoids were equally effective in protection from photooxidation.

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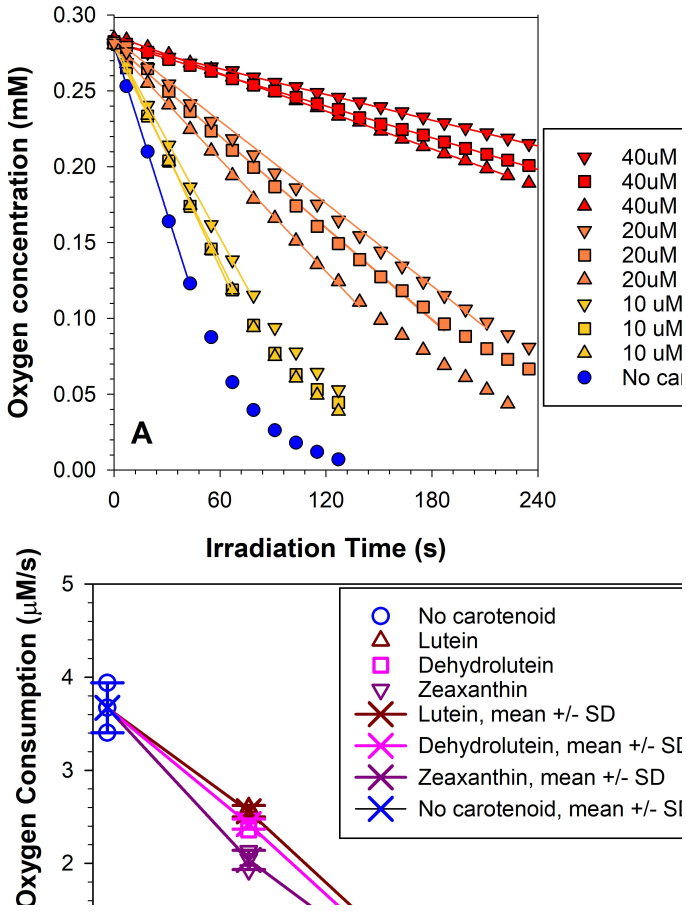

Figure 3. Dehydrolutein inhibits all-*trans*-retinal-mediated photooxidation with similar efficiency to lutein and zeaxanthin. A) Representative kinetics of oxygen consumption during exposure of liposomes to all-*trans*-retinal and blue light in the absence and presence of carotenoids at indicated concentrations. Liposomes, consisting of 2.5 mg/ml EYPC, 0.75 mM all-*trans*-retinal, and indicated concentration of carotenoids, were exposed to blue light (404 ± 6 nm; 8.1 mW/cm^2) in the presence of 1% DMSO. The samples included 0.1 mM mHCTPO used as a spin probe. The initial linear portions of the kinetics were fitted to straight lines, and the slopes of these lines gave the initial rates of oxygen consumption. B) The initial rates of oxygen consumption in the absence and presence of indicated carotenoids as a function of carotenoid concentration. Statistically significant differences between the initial rates of oxygen consumption were observed between the carotenoid at 10 μM concentrations only; oxidation in the presence of 10 μM zeaxanthin was significantly slower than for 10 μM dehydrolutein or lutein ($p<0.001$, in both cases).

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1.2. Effects of supplementation with dehydrolutein, lutein, and zeaxanthin on carotenoid content in cultured retinal pigment epithelial cells ARPE-19

ARPE-19 cells are a spontaneously immortalised cell line derived from retinal pigment epithelium of 19-year-old male donor, and were used in passage numbers where these cells retain their retinal pigment epithelial cell characteristics [46]. Importantly, these cells express receptors, such as SR-BI and LDL receptors, which have been demonstrated to be involved in carotenoid uptake [58-60]. To compare the effects of dehydrolutein with those of lutein and zeaxanthin in cultured ARPE-19 cells, the confluent cells were incubated for up to 19 days with 2 μM carotenoids solubilized first in foetal calf serum (FCS) and then in the culture medium to mimic physiological conditions where carotenoids are carried in the blood plasma mostly bound to lipoproteins. The concentration of carotenoids was chosen based on the highest values of lutein and zeaxanthin in human serum reported in literature. At the time when lutein and zeaxanthin supplements were not available, it was determined, in a large cohort of 8,229 persons above the age of 40 years of various ethnic backgrounds, that the average combined concentration of lutein and zeaxanthin in human serum in people with the highest values was 0.79 μM [61]. It was shown later that by supplementation these values can be increased to about 2 μM [12,62,63].

At selected days, cells were trypsinized, 40 μl aliquots were taken for determination of cell concentration, and the remaining suspension was used for extraction of carotenoids and their quantification by spectrophotometry (Fig. 4). Feeding cells with zeaxanthin resulted in a monotonic, almost linear increase in concentration of cellular zeaxanthin, reaching concentrations of about 1.1 nmol/million cells after 8 feedings provided over 19 days (Fig. 4). Lutein uptake was less efficient and appeared to reach a plateau after 6 feedings, and a cellular content of about 0.7 nmol/million cells after 19 days. This is consistent with previous studies showing that ARPE-19 cells accumulate lutein and zeaxanthin and can achieve intracellular concentrations exceeding up to several-fold the concentration of these carotenoids in the culture medium [60,64]. In contrast, cells fed with dehydrolutein accumulated very little, if any, of this carotenoid (Fig. 4). It has been suggested that any dehydrolutein formed in the retina by oxidation of lutein may be reduced to regenerate lutein or epi-lutein [3]. Reduction of keto carotenoid canthanxanthin to its corresponding mono- and diol derivatives has been documented in the primate retina [65]. However, we did not observe accumulation of any carotenoids in cells fed with dehydrolutein. These results indicate a highly selective uptake and accumulation of lutein and zeaxanthin, and a surprising ability of RPE cells to either exclude or efficiently excrete absorbed dehydrolutein.

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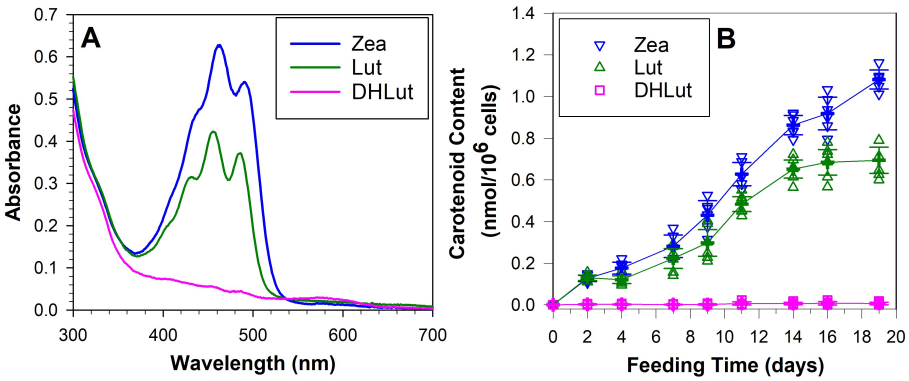

Figure 4. ARPE-19 cells accumulate lutein and zeaxanthin but not dehydrolutein. A) Representative absorption spectra of extracts from ARPE-19 cells supplemented for 19 days with cell culture medium containing 10% FCS enriched with 2 μM lutein, dehydrolutein, or zeaxanthin. B) Concentrations of lutein, zeaxanthin, and dehydrolutein in ARPE-19 cells fed for indicated number of days with culture medium enriched with 2 μM carotenoids.

1.3. Effects of supplementation with dehydrolutein, lutein, and zeaxanthin on viability of ARPE-19 cells

Importantly, the supplementation with carotenoids did not affect cell numbers in the confluent cultures: the cell numbers remained similar over the entire period of supplementation in the flasks fed with different types of carotenoids, similar to cell numbers in the flasks treated with DMSO only, and similar to cell numbers per flask prior the supplementation treatment (Fig. 5A). Interestingly, the reductive activities of carotenoid-containing cells were significantly reduced in comparison with cells fed with dehydrolutein or DMSO (Fig. 5B). The reductive activity of cells fed with lutein or zeaxanthin, measured by the MTT assay, were 18 and 19% smaller, respectively, than reductive activitie of cells supplemented with DMSO only ($p<0.001$ in both cases). Cells fed dehydrolutein had a decreased reductive activity that was 8% lower than cells fed with DMSO but that difference was not statistically significant ($p=0.075$).

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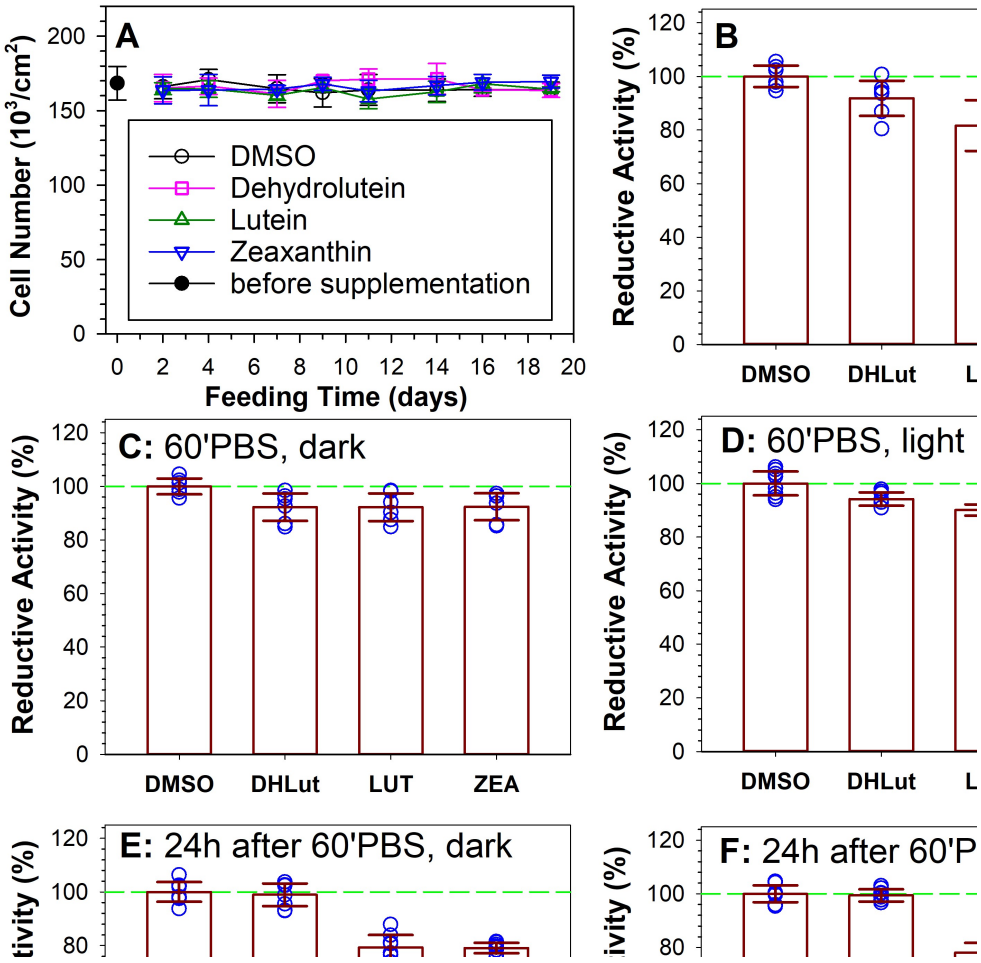

Fig. 5. Supplementation with carotenoids does not affect cell viability but dehydrolutein can decrease their reductive activities. A: ARPE-19 cell density before and after feeding with carotenoids for up to 19 days. The symbols represent means, the error bars represent SDs. B: Reductive activities of cells after 19 days of supplementation with vehicle (DMSO) or carotenoids: dehydrolutein (DHLut), lutein (LUT), and zeaxathin (ZEA) measured by MTT assay. C,D: Reductive activities of cells after 19 days of supplementation with vehicle (DMSO) or indicated carotenoids measured by MTT assay after 1 h incubation in Dulbecco's phosphate buffered saline (PBS) in dark (C) or with concomitant exposure to white light (D). E, F: Reductive activities of cells after 19 days of supplementation with vehicle (DMSO) or indicated carotenoids measured by MTT assay 24 hours after 1 h incubation in PBS in dark (E) or with exposure to white light (F). B-F: the symbols correspond to individual measurements, the heights of the bars correspond the means, the error bars correspond to SDs.

The 1 h exposure of ARPE-19 cells to Dulbecco's phosphate buffered saline (PBS), in the presence or absence of visible light, upregulated the reductive activities of cells fed lutein or zeaxanthin so the values were similar for all three carotenoids (Fig. 5 C, D), suggesting that deprivation of glucose and other nutrients present in the normal growth media can upregulate the reductive activity once they are available again. Twenty four hours post-exposure to Dulbecco's PBS with and without light, the reductive activities of lutein-

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or zeaxanthin-containing cells returned to the values similar to those observed without any pre-treatment in PBS (Fig. 5 B, E, F). It has been shown previously on canine lens epithelial cells that lutein can decrease their reductive activities measured by MTT assay [66]. The reductive activity assay measured by MTT reflects mainly the activity of glycolytic enzymes and NAD(P)H production [67]. It has been shown that lutein can decrease activities of several reductive enzymes, such as aldose reductase, sorbitol dehydrogenase, isocitrate dehydrogenase [68,69], and that lutein and zeaxanthin can decrease the rate of oxidative phosphorylation [70-74]. However, there are also reports demonstrating that lutein can increase glycolysis and activities the oxidative phosphorylation [75]. Because the presence of carotenoids affected the reductive activities of cells, the reductive activities of cells exposed to photosensitizers and light were calculated as a ratio of reductive activity of cells exposed to light in the presence of the photosensitizer to reductive activity of cells in the absence of the photosensitizer, but subjected to the same treatment with respect to carotenoids and light.

1.4. Effects of supplementation with dehydrolutein, lutein and zeaxanthin on susceptibility of ARPE-19 cells to photosensitized damage

Similarly to a previous report [47], exposure to green light in the presence of rose bengal induced a rapid damage to ARPE-19 cells resulting in an immediate decrease in their reductive activity (and viability) when measured immediatedly after the 1 hour exposure (Fig. 6A). The reductive activity in cells treated with DMSO without carotenoids and exposed to rose bengal and light decreased by 76% in comparison to cells exposed to light in the absence of rose bengal. The reductive activities of cells fed for 19 days with lutein, zeaxanthin, or dehydrolutein and then exposed to rose bengal and light were similar to that in cells without carotenoids (Fig. 6A).

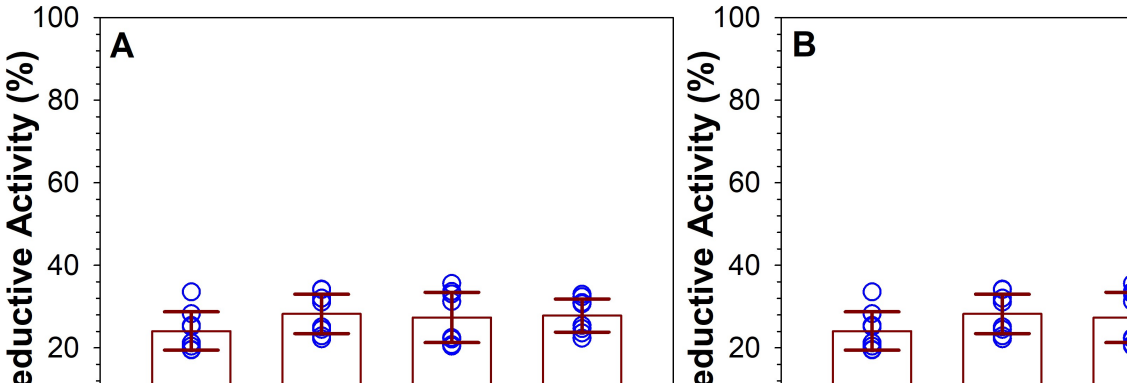

Fig. 6. Supplementation with carotenoids for 19 days does not affect reductive activity of cells following damage induced by photosensitizers and light. Reductive activities of cells after 19 days of supplementation with vehicle (DMSO) or carotenoids: dehydrolutein (DHLut), lutein (LUT), and zeaxanthin (ZEA) measured by MTT assay immediately after 1 h exposure to visible light (0.46 mW/cm^2) and 0.5 μM rose bengal (A) or 24 hours after 1 h exposure to visible light and liposomes containing 0.5 mM all-*trans*-retinal and 2.8 mg/ml EYPC (B). The reductive activities are expressed as ratios of reductive activity of cells in the presence of the photosensitizer to reductive activity of cells exposed to the same treatment in the absence of the photosensitizer. The symbols

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indicate individual measurements, the heights of the bars indicate the means, and the error bars indicate SDs. There were no statistically significant differences between any of the four treatments with rose bengal or all-*trans*-retinal.

Unlike phototoxicity of rose bengal, which causes immediate loss of cell viability [47], all-*trans*-retinal phototoxicity takes longer to affect cell viability [19], and therefore the reductive activities were measured 24 hours after the exposure. The reductive activities of cells exposed to all-*trans*-retinal and light were decreased in comparison to cells without all-*trans*-retinal by about 78% (Fig. 6B). Responses of cells that were supplemented with lutein, zeaxanthin, dehydrolutein, or DMSO only were not significantly different from each other. Interestingly, the carotenoids accumulated over 19 days of feeding did not make cells more resistant to photosensitized killing by either rose bengal or all-*trans*-retinal when compared to cells without carotenoids (Fig. 6A,B). It appears that incorporation of lutein and zeaxanthin into cells makes them ineffective in protecting the cells from photosensitizers which generate their reactive oxygen externalto the plasma membranes.

To determine whether carotenoids can protect the cells against photodamage while localised extracellularly, cells pre-treated with carotenoids for 19 days were exposed to rose bengal and light in the presence of 2 μM carotenoids and 0.2% DMSO solubilized directly in Dulbecco's PBS (Fig. 7A). This treatment increased the reductive activity from 25% in DMSO-treated cells to 40, 41 and 38% for cells supplemented with dehydrolutein, lutein, or zeaxanthin, respectively. This approach also proved effective in protection against photodamage induced by all-*trans*-retinal and light, where carotenoids increased reductive activities from 25% in DMSO-only-treated cells to about 36, 34, and 33%, respectively, for cells supplemented with lutein, zeaxanthin, or dehydrolutein (Fig. 7B). There were no statistically significant differences between any of the three carotenoids.

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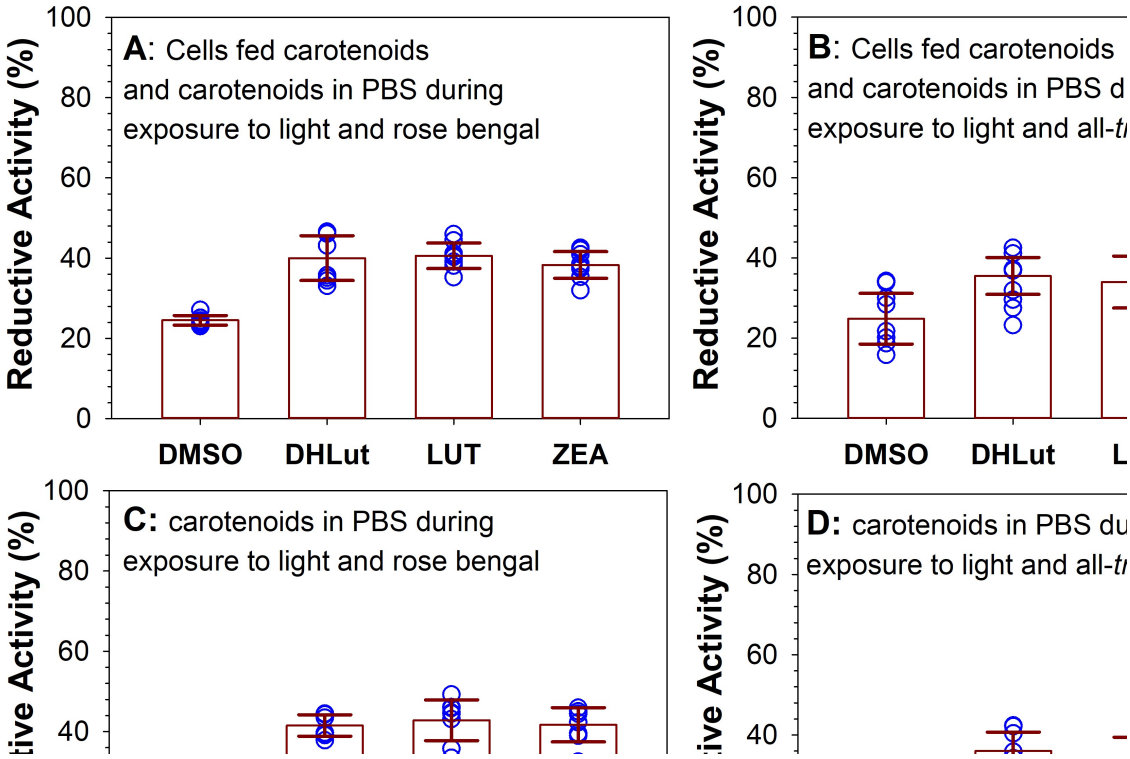

Fig. 7. The presence of extracellular carotenoids partly protects cells from damage induced by photosensitizers and light independently of whether or not the cells underwent prior supplementation with carotenoids. A, B: Reductive activities of ARPE-19 cells after 19 days of supplementation with vehicle (DMSO) or carotenoids: dehydrolutein (DHLut), lutein (LUT), and zeaxathin (ZEA) measured by MTT assay immediately after 1 h exposure to visible light and 0.5 μM rose bengal (A) or 24 hours after 1 h exposure to visible light and liposomes containing 0.5 mM all-*trans*-retinal and 2.8 mg/ml EYPC (B). The exposure to photosensitizers and light was in the presence of 0.2% DMSO and 2 μM carotenoids solubilised in DPBS. C, D: Reductive activities of cells without prior feeding with carotenoids, which were exposed to light, photosensitizers and carotenoids and the MTT assay was performed immediately after 1 h exposure to light and 0.5 μM rose bengal (C) or 24 hours after 1 h exposure to light and all-*trans*-retinal (D). The exposure to photosensitizers and light was in the presence of 0.2% DMSO and 2 μM carotenoids solubilised in DPBS. The symbols indicate individual measurements, the heights of the bars indicate means and the error bars indicate SDs. Treatments with carotenoids resulted in a statistically significant increase in the reductive activities in comparison with DMSO-only-treated cells in all four types of experiments ($p<0.001$). The differences between effects of different carotenoids in A, B, C and D were not statistically significant.

There are numerous reports showing that lutein and/or zeaxanthin can modulate expression of several antioxidant enzymes, and can activate the nuclear factor erythroid 2-related factor 2 (Nrf2) signalling responsible for the expression of antioxidant response element (ARE) genes, and subsequently, the synthesis of antioxidant and detoxification proteins [68,69,76-89]. It has been also reported that DMSO can activate Nrf2 [90]. Therefore, we considered a possibility that long-term feeding with carotenoids/DMSO may affect cellular responses to oxidative stress induced by exposure to light and photosensitizers, and used ARPE-19 cells without the long-term feeding with carotenoids

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and exposed them to photosensitizers and light with DMSO/carotenoids provided at the same time (Fig. 7 C,D). Under these conditions, the reductive activities were increased from about 22% to 42, 43, or 42% for cells exposed to light and rose bengal, and from 20% to 36, 35 and 32% for cells exposed to light and all-*trans*-retinal in the presence of dehydrolutein, lutein, or zeaxanthin, respectively (Fig. 7 C,D). Again, in none of these cases were significant differences in protection offered by the three carotenoids.
The protection offered by the short-term treatment with carotenoids was similar in cells with and without long-term carotenoid supplementation (Fig. 7 A,B,C,D), so it appears that the accumulation of carotenoids inside cells does not affect cellular responses to the photooxidative damage induced by extracellular photosensitizers and light.

4. Discussion

Altogether, our results demonstrate that antioxidant properties of dehydrolutein are similar to those of lutein and zeaxanthin: all three carotenoids are effective singlet oxygen quenchers, which can slow down photooxidation of lipids mediated by photoexcitated rose bengal or all-trans-retinal. It may be inferred that because lutein and zeaxanthin are sequestered in the retina bound to their binding proteins with high specificity and selectivity [8,49-53], their diffusion rates [54], and consequently their efficiency in protectiing lipid membranes from damage induced by singlet oxygen and other reactive oxygen species may be limited, particularly within cellular memvranes. On the other hand, to date no dehydrolutein-binding protein has been identified. Therefore, the localisation of dehydrolutein in the retina deserves further investigation to determine i) in which retinal layers, ii) in which cell types and their subcellular compartments it is present, and iii) whether it is present as a free carotenoid in lipid membranes or it is bound to proteins.

Our results also demonstrate that a long-term exposure of ARPE-19 cells to dehydrolutein, lutein, or zeaxanthin does not affect cell viability, leads to a substantial accumulation of lutein and zeaxanthin within the cells, but dehydrolutein does not accumulate to a significant extent. This interesting observation suggests that ARPE-19 cells possess a selective uptake mechanism for lutein and zeaxanthin but can exclude dehydrolutein or, alternatively there is an efficient mechanism of dehydrolutein removal following uptake. It has been shown that ARPE-19 cells can take up lutein and zeaxanthin from human serum, and the uptake is more efficient for these carotenoids when zeaxanthin is bound to HDLs than to LDLs, and when lutein is bound to LDLs than to HDLs [60]. In the human retina, similar to the results shown here, the selective accumulation of occurs. Of about 14 dietary carotenoids present in blood serum [91], only two accumulate in the retina [11-15]. The RPE, together with endothelial cells of retinal capillaries, provides the blood-retina barrier, and therefore it is involved in the selective uptake of carotenoids and their transepithelial transport [59]. However, the mechanisms responsible for that selectivity and transepithelial transport into the retina are still poorly understood. Further investigations of dehydrolutein interactions with ARPE-19 cells may be helpful as a model to investigate these transport mechanisms and determine if the presence of dehydrolutein in the retina is a result of its uptake from the blood via transepithelial transport or a result of conversion of lutein/zeaxanthin inside the retina.

Interestingly, despite accumulation of substantial amounts of lutein and zeaxanthin, none of these carotenoids protects ARPE-19 cells from photooxidative damage caused by photosensitizers which are present external to the cells – either as water soluble, cell-impermeable rose bengal or lipophyllic all-trans-retinal incorporated into lipid vesicles. Partial protection from photooxidative damage induced by extracellular photosensitizers is achieved by all three carotenoids, each having similar efficiency, but only when they are present extracellularly together with the photosensitizers. These findings underline the importance of co-localisation of antioxidants with potential sources of reactive oxygen species. Some previous studies also indicate that zeaxanthin and/or lutein can

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exert protection in cultured ARPE-19 cells against photooxidative damage, and that protection is more efficient if carotenoids are co-localized with photosensitizers [92-95]. Wrona et al. have shown that zeaxanthin supplemented to ARPE-19 cells exposed to visible light and merocyanine 540, a well- characterized photosensitizer which associates with cell membranes, can substantially diminish oxidation of cholesterol but does not offer a significant protection against cytotoxicity unless it is given in combination with vitamin C or vitamin E and the exposure to photooxidation is short [92]. In those studies zeaxanthin was administered to ARPE-19 cells 24 hours prior to exposure to the photosensitizer and light. In other studies, where the photosensitizers were present inside the cells, such as phagocytosed lipofuscin, melanolipofuscin, or melanosomes, a combination of zeaxanthin with vitamin E were, at least in part, preserving cell viability [93-95]. In these experiments, pigment granules were enriched with antioxidants prior to administration to cultured cells. However, lutein and zeaxanthin do not accumulate in substantial concentrations in the human RPE [34,35].

The photoreceptor outer segments are the main layers in the retina where lutein and zeaxanthin are detected in substantial concentrations [34,35]. Potent photosensitizers can transiently accumulate in these parts of neurons, and upon absorption of light generate singlet oxygen [19-21,37]. In photoreceptor outer segments all-trans-retinal is hydrolysed from photoactivated visual pigments, and its clearance is dependent mainly on activities of NADPH-dependent retinol dehydrogenase and ATP-binding cassette transporter rim protein (ABCR, also known as ABCA4) [19,21,36,56,57,96]. The loss of function mutations in genes coding these enzymes result in delayed clearance of all-trans-retinal and increased susceptibility to light-induced damage to the retina in the mouse. In the human retina, mutations in ABCA4 gene can lead to Stargardt's disease, as well as are associated with increased risk of certain subtype of retinitis pigmentosa and age-related macular degeneration. It is believed that photoexcitation of all-trans-retinal leads to generation of singlet oxygen and initiates the damage. Photoreceptor outer segments are rich in polyunsaturated fatty acids, such as docosahexaenoic acid, which are very susceptible to oxidation by singlet oxygen and subsequent formation of lipid hydroperoxides. Lipid hydroperoxides may themselves undergo decomposition in the presence of iron ions. Iron ions are known to accumulate with age in the retina and that accumulation is increased in age-related macular degeneration. Decomposition of lipid hydroperoxides generate lipid peroxyl radicals, which can propagate lipid peroxidation, resulting in the formation of end-products with photosensitizing properties [97]. Because these photosensitizing species are not distributed uniformly and are likely to be localized in lipophilic structures, it is important to determine to what extent carotenoids present in the photoreceptor outer segments are free and localized in the lipid membranes or are bound to proteins in the cytosolic environment. The localization of dehydrolutein that is present within the retinaremains incompletely investigated. Its presence in the lipid membrane would be significant given its ability to function as a photoprotective agent.

In conclusion, our findings point to the importance of determining whether dehydrolutein is present in the retina as a free carotenoid in lipid membranes or bound to proteins. In order to elucidate the role of RPE in selective carotenoid transport further investigation of the mechanism of transport should be untaken. This information will provide insight and enable further development of therapeutic strategies for increasing carotenoid accumulation in lipid membranes of the RPE and photoreceptor outer segment membrane where they are needed the most as singlet oxygen quenchers and can most effectively can protect against light-induced injuries which can contribute to development of age-related macular degeneration.

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