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Concentration-Dependence of Vitamin C in Combinations with Vitamin E and Zeaxanthin on Light-Induced Toxicity to Retinal Pigment Epithelial Cells†

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Abstract

The purpose of this study was to determine the effects of increasing concentration of ascorbate alone and in combinations with α -tocopherol and zeaxanthin on phototoxicity to the retinal pigment epithelium. ARPE-19 cells were exposed to rose bengal and visible light in the presence and absence of antioxidants. Toxicity was quantified by an assay of cell reductive activity. 20 min exposure to visible light and photosensitizer decreased cell viability to ~42%. Lipophilic antioxidants increased viabilities to ~70%, ~61% and ~75% for α -tocopherol, zeaxanthin and their combination, respectively. Cell viabilities were ~70%, 56% and 5% after exposures in the presence of 0.35, 0.7 and 1.4 mM ascorbate, respectively. 45 min exposure increased cell death to ~74% and to >95% in the absence and presence of ascorbate, respectively. In the presence of ascorbate, zeaxanthin did not significantly affect phototoxicity. α -Tocopherol and its combination with zeaxanthin enhanced protective effects of ascorbate but did not prevent from ascorbate-mediated deleterious effects. In conclusion, there is a narrow range of concentrations and exposure times where ascorbate exerts photoprotective effects, exceeding which leads to ascorbate-mediated increase in photocytotoxicity. Vitamin E and its combination with zeaxanthin can enhance protective effects of ascorbate but do not ameliorate its deleterious effects.

INTRODUCTION

The retina, and particularly its outermost part - retinal pigment epithelium (RPE) is constantly at risk of photooxidative stress due to daily exposures to visible light, presence of potent photosensitizers, and high oxygen tension (1). RPE is a monolayer of cells separating the retinal photoreceptors from the blood supply from the fenestrated choriocapillaris (2, 3). RPE cells provide the blood-retina barrier and are responsible for transport of nutrients and waste products between the blood and the photoreceptive part of the retina, including antioxidants of dietary origin such as ascorbate (vitamin C), α -tocopherol (vitamin E) and carotenoids (2). Interestingly, out of about 16 different carotenoids normally present in the blood plasma, only zeaxanthin, lutein and lutein metabolite – *meso*-zeaxanthin accumulate in the retina.

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It has been demonstrated that vitamins C and E and carotenoids, administered as single antioxidants or in combinations, can partly protect biomolecules or RPE cells from oxidative damage due to their ability to scavenge free radicals and/or quench electronically excited state of molecular oxygen O_2 ($^3\Sigma_g^-$) known as singlet oxygen ($^1\Delta_g$, 1O_2) (4–14). In particular, it has been demonstrated that combinations of zeaxanthin with vitamin C or zeaxanthin with vitamin E can offer synergistic protection of cultured RPE cells from photooxidative damage, where single antioxidants did not offer a significant protection (10). The mechanisms responsible for the synergistic protection involve protection of zeaxanthin from free radical-mediated degradation by ascorbate or α -tocopherol so zeaxanthin can act longer as a singlet oxygen quencher. However, this synergistic protective effect was present only at short irradiation times of 15 and 30 minutes. After 60 min irradiation there was no significant protective effect of antioxidants on cell viability. At this time point above 50% of zeaxanthin was degraded. The concentration of ascorbate used in this study was 0.5 mM, which is within the physiological range. However, in neural tissues ascorbate accumulates in millimolar concentrations (15, 16). Thus it can be suggested that antioxidant protection can be increased by increasing the concentration of ascorbate.

On the other hand, ascorbate can exert pro-oxidant effects by interacting directly with a photoexcited photosensitizer or singlet oxygen leading to production of free radicals and hydrogen peroxide (17, 18). There are several reports where ascorbate enhances photodynamic damage, particularly in the presence of iron (10, 19–25). This is important because RPE cells are constantly involved in trafficking of iron between the photoreceptors and choroidal blood supply (26, 27). Moreover, there is a substantial increase with age in weakly chelated iron ions in the human RPE-choroid complex and that increase is further enhanced in people affected by age-related macular degeneration (AMD) (28–30). AMD is a multifactorial disease which is the major cause of blindness in the elderly in developed countries (31–33). Thus it remains an intriguing question whether anti-oxidant properties of ascorbate outweigh its pro-oxidant properties in the aged RPE, particularly when it is affected by AMD. This is likely to be dependent on the concentration of ascorbate and therefore finding the optimal concentration of ascorbate to be protective is essential.

In an attempt to determine the optimal concentration of ascorbate and its effects in combinations with zeaxanthin and α -tocopherol, we employed cultures of ARPE-19 cells, a spontaneously immortalized cell line derived from the RPE of a 19-year old human male donor, which exhibit similar susceptibility to oxidative stress as primary RPE cultures (34). ARPE-19 cells were exposed to photooxidative stress induced by green light and rose bengal (RB) in the absence and presence of physiologically-relevant concentrations of ascorbate, zeaxanthin, α -tocopherol and combinations thereof. Cell viability was assessed by morphology and quantified by an assay of cellular reductive activity.

MATERIALS AND METHODS

RPE cell culture

Spontaneously immortalized cell line ARPE-19 was obtained after 21st passage from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in a GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, GIBCO, UK) supplemented with antibiotics (0.1 mg/mL streptomycin, 0.1 mg/mL kanamycin and 0.06 mg/mL penicillin; Sigma, UK), Fungizone (1.25 µg/mL Amphotericin B; GIBCO, UK) and 10% v/v heat-inactivated fetal calf serum (FCS; BioSera, UK). DMEM/F12 is the recommended culture medium by the ATCC and contains redox-active metal ions: 1.5 µM ferric sulphate and 0.124 µM ferric nitrate. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂ and 95% air and medium was changed every 2–3 days (3 times a week). After reaching confluency, the concentration of FCS was

decreased to 2%. For selected experiments cells were cultured as above but DMEM/F12 was replaced by Minimal Essential Medium Eagle (MEM; Sigma-Aldrich) which is not supplemented in iron salts and cells were passaged at least three times on MEM-based cultured medium before sub-culturing into 24-well plates for experiments.

Exposure of ARPE-19 cells to rose bengal and green light in the presence and absence of antioxidants

For light-induced cytotoxicity experiments, cells from 26th to 29th passage were used and cells were cultured in 24-well plates. After reaching confluency, cells were maintained in 1ml/well culture medium with 2% FCS for two media changes (corresponding to 4–5 days) to minimize variability of responses to photooxidative stress due to differences in cell densities between experiments (35). Immediately before exposure to RB and light, the culture medium was removed and the wells were gently washed twice with phosphate buffered saline with an addition of calcium chloride and magnesium chloride (Dulbecco's PBS, GIBCO, UK; D-PBS). ARPE-19 cells were exposed to 1 ml/well of solutions in D-PBS containing selected concentration of RB in the presence 0.2% dimethylsulfoxide (DMSO) needed for solubilisation of the lipophilic antioxidants with or without selected concentrations of zeaxanthin (DSM Nutritional Products AG, Switzerland), α -tocopherol and ascorbic acid (Sigma-Aldrich) and combinations thereof. Concentrations of antioxidants were selected based on available literature on physiological concentration of antioxidants in relevant tissues and/or effective concentrations in previous experiments on cultured cells. Ascorbate was tested at concentrations up to 2.0 mM, zeaxanthin - at 2 μ M, and α -tocopherol – at 50 μ M (10, 16, 36).

Culture plates with cells in the presence of 0.05–1.00 μ M RB, antioxidants or the vehicle (0.2% DMSO) solubilized in D-PBS were exposed for selected times to visible light (mainly green) from a solar simulator Sol lamp (Hönle UV Ltd, Birmingham) with a set of filters placed between the lamp and the glass shelf where the cell culture plate was positioned: heat filter (Lee Heat Shield; Lee Filters, UK), UV filter (#226 Lee UV filter; Lee Filters, UK) and a green filter (#090 “dark yellow green”; Lee Filters, UK). Spectral irradiance of the light source was measured in each experiment using a handheld spectroradiometer (Specbos 1201 with JETI LiMeS software; Glen Spectra, UK). The distance between the lamp and surface of the culture plate was adjusted to provide irradiance of ~ 2.8 mW/cm² (Supplemental Fig. S1). During exposure to light, the temperature was held at $\sim 34^\circ\text{C}$. The rates of generation of singlet oxygen by photoexcited RB in wells with cultured cells were calculated as a product of singlet oxygen quantum yield of 0.75 and the integrated flux rates of absorbed photons (37, 38; Supplemental Fig. S1). The spectral flux rates of absorbed photons N_a were calculated for each wavelength λ from the range of 390 to 780 nm using the formula:

$$N_a = \lambda I_0 (1 - 10^{-A}) / (hc)$$

where I_0 stands for irradiance at λ ; A – absorbance at λ in culture wells with 1 ml 250 nM RB solution over cultured cells providing optical pathlength of 0.52 cm; h – Planck's constant; c – speed of light in vacuum. To express the spectral flux rates of absorbed photons in einsteins, the N_a was divided by the Avogadro's number.

For incubations with RB/antioxidants in dark, culture plates were wrapped in blackened aluminium foil and then in aluminium foil, and placed above the lamp to mimic the temperature and air conditions experienced by light-exposed cells. After exposure to RB/light cells were washed twice with D-PBS, covered with culture medium containing 2% FCS and returned to the incubator until cytotoxicity essays.

Phase contrast microscopy for imaging of cell monolayer morphology

ARPE-19 cells were visualized using an Olympus IX70 inverted microscope with phase contrast. Images were obtained using a Spot RT colour CCD camera and Spot Advanced software (Diagnostic Instruments Inc, UK).

Assay of reductive activity of cells

Unless stated otherwise, 1 h after exposure to RB/light ARPE-19 cells were incubated for 1 hour with 1 ml/well of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (39, 40). Then, culture medium with MTT solution was aspirated, cell monolayers were washed with D-PBS and after aspirating D-PBS, formazan crystals were dissolved in acidified isopropanol (200 μ l/well of isopropanol with 0.04 M HCl). Accumulated formazan was quantified by its absorbance at 590 nm. Absorbance at 690 nm was used as a measure of light scatter and absorbance values at 590 nm were corrected. Background measurements were taken in cells exposed to acidified isopropanol only (i.e. no RB and no MTT) and corresponding absorbance values were subtracted.

For experiments with multiple time-points, the reductive activity was expressed as a ratio to control cells from the same plate (expressed as percentage) which were subjected only to 0.2% DMSO in D-PBS for the identical incubation periods as cells treated with RB/antioxidants. At least two wells in each plate were exposed to the same treatment. All experiments were repeated at least three times.

In a set of control experiments the reductive activities of cells incubated in dark or exposed to visible light in the presence of 0.2% DMSO were compared.

Data analysis

Data analyses were carried out using Microsoft Office Excel 2007 and SigmaPlot for Windows version 11.0. Unless stated otherwise, data points in graphs represent the means, while error bars – SDs calculated from all individual wells which underwent the same treatment. Statistical comparisons were carried out using SigmaPlot 11.0 by one way analysis of variance (ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method).

RESULTS

Effects of rose bengal (RB) and green light on ARPE-19 cell viability: dependence on RB concentration, exposure time and post-exposure time

RB when photoexcited with green light induces photooxidative damage mediated by production of singlet oxygen and to a smaller extent - free radicals (9, 41–44). As a result, RB can cause apoptosis (45) and/or damage components of the plasma membrane (46–48). Before testing effects of antioxidants on phototoxicity, the concentration of RB, exposure times and assessment of toxicity were optimised. Cells exposed to visible light in the presence of low concentrations of RB ($\leq 0.1 \mu$ M) showed no major morphological changes when observed by phase contrast microscopy (Fig. 1). At increasing concentrations, considerable changes in morphology were evident, with cell shrinkage (resulting in highly refractile cells) and some cell detachment observed. Cell loss from the monolayer was apparent after exposure to visible light in the presence of 0.2μ M RB. However, at higher concentrations of RB, more and more cells remained adherent to the bottoms of culture wells even though their reductive activity was decreased $>95\%$ (Fig. 1,2,3). At the highest concentration tested of 1μ M, all cells appeared attached, although they were completely different in appearance to unaffected cells – with cells becoming more spindle shaped, and often with pronounced nuclei. This effect of RB is likely due to photo-induced formation of

cross-links by photoactivated rose bengal (49–54) and indicates that the quantification of cell viability based on density of cells remaining in the monolayer is not feasible and can produce artefactual results.

Reductive activity of cells was significantly decreased immediately after 1 h exposure to visible light in the presence of 200 nM RB and higher concentrations (Fig. 2a). An exposure to light in the absence of RB or exposure to 1 μ M RB in the dark did not affect cell morphology (data not shown) or their reductive activities (Fig. 2b). Expanding the time between the end of exposure and MTT assay led to a further time-dependent decrease in reductive activity of cells exposed to both RB and light (Fig. 2a). Because there were no substantial changes in reductive activities between 1 and 2 h post-exposure times, 1 h post-exposure was selected for the beginning of the MTT assay for all further experiments in the presence and absence of antioxidants.

The phototoxic effects of RB increased not only for increasing concentrations of RB but also with increasing exposure times (Fig. 3). Because 250 nM RB exerted substantial phototoxic effects already after 20 min exposure and reductive activity of cells was still measurable after 90 min exposure, 250 nM RB was selected for further assays in the presence and absence of antioxidants.

The calculation of the integrated rates of photon absorption by 250 nM RB in culture wells gave value of 113×10^{-12} einstein $s^{-1} cm^{-2}$ (Supplemental Fig. S1) and therefore the estimated fluxes of singlet oxygen generated during exposure of cells light were $85 pmol s^{-1} cm^{-2}$ providing a flux of singlet oxygen of 162 nM/s when averaged over the entire volume of 1 ml RB solution in a well with $1.91 cm^2$ surface. This value appears to be within a physiological range of singlet oxygen generated *in vivo* in the aged human RPE where lipofuscin occupies 19% of the cytoplasmic volume and it is exposed to bright daylight when up to $0.1 mW/cm^2$ of visible light is incident on the retina (38, 55, 56). If, under these conditions, lipofuscin was solubilized in the RPE, the rate of photon absorption would be at least 139×10^{-12} einstein $s^{-1} cm^{-2}$ and the rate of generation of singlet oxygen would be at least $7 pmol s^{-1} cm^{-2}$. Considering 14 μ m thickness of RPE cells, it would give at least 5 μ M/s of singlet oxygen produced in the RPE layer - a value which is 31-fold greater than the singlet oxygen flux produced in our experiments. It needs to be considered, however, that lipofuscin is enclosed within granules and therefore the absorption cross-section may be much smaller than under conditions when it is solubilised. Also, in these considerations we ignored the insoluble part of lipofuscin which in the old eyes contributes to lipofuscin photoreactivity even more than the soluble part and therefore may contribute to an increase of singlet oxygen flux (57).

Effects of different concentrations of ascorbate on RB-mediated photocytotoxicity

The effects of ascorbate on phototoxic effects of RB to ARPE-19 cells cultured in DMEM/F12 were strongly concentration dependent (Fig. 4). Vitamin C exerted the greatest protective effect at the smallest concentration tested of 0.35 mM and 20 min irradiation time by increasing cell viability from ~42% to ~70% ($P < 0.001$). Doubling the concentration of ascorbate to 0.7 mM during 20 min irradiation resulted in a decrease in protection in comparison with 0.35 mM ascorbate but the increase in cell viability to ~56% was still significant in comparison to cells without ascorbate. In the presence of 1.4 mM ascorbate during 20 min irradiation time, cell viability decreased to only ~5% which was significantly smaller than for cells without ascorbate ($P < 0.001$). Extending the irradiation time to 45 min resulted in a decrease of cell viability to ~26% in the absence of ascorbate, whereas in the presence of any of the three concentrations of ascorbate, cell viability decreased to less than ~4% ($P < 0.001$). Exposure to light in the absence of RB resulted in no significant changes in cell viability irrespectively of ascorbate concentration or irradiation time.

To determine whether the increase in phototoxicity was related to iron ions from iron-rich culture medium DMEM/F12, the effects of various concentrations of ascorbate on phototoxic effects of RB were also evaluated for cell cultured in MEM which is not supplemented with iron. Interestingly, for cells cultured in MEM, ascorbate did not exacerbated photocytotoxic effects of RB even at the highest concentrations tested of 2 mM (Supplemental Fig. S2). In contrast, its protective effect monotonically increased with the increasing concentration but the differences were not statistically significant in comparison with cells without ascorbate.

Effects of zeaxanthin, α -tocopherol and their combination on RB-mediated photocytotoxicity

In case of ARPE-19 cells cultured in DMEM/F12, zeaxanthin alone exerted statistically significant protective effects on RPE survival for 20 and 45 minutes irradiation times, increasing cell viability from 42% to 61% and from 26% to 43%, respectively ($P < 0.001$) (Fig. 5). The protective effect of zeaxanthin was no longer present for 90 min irradiation time ($P = 0.469$). Vitamin E exerted even greater protective effects than zeaxanthin increasing cell viability to 70% and 59% for 20 and 45 min irradiation times, respectively ($P < 0.001$ for both). In contrast to zeaxanthin, α -tocopherol exerted protective effects also for 90 min irradiation time increasing cell viability from 12% to 22% ($P < 0.001$).

A combination of zeaxanthin with α -tocopherol exerted slightly greater protection than α -tocopherol alone but the increase reached statistical significance only for 90 min irradiation ($P = 0.013$).

The effects of zeaxanthin and α -tocopherol on cells cultured in DMEM/F12 were largely similar to the effects exerted on cells cultured in MEM (Fig. 5, Supplemental Fig. S3). For cells cultured in MEM, zeaxanthin increased cell viability from 30% to 40%, α -tocopherol – to 47%, whereas the combination of zeaxanthin with α -tocopherol – to 61% (Fig. S3). Here, the combination of antioxidants exerted significantly greater protection than either zeaxanthin or α -tocopherol alone. Moreover, the survival of 61% of cells was the same within experimental uncertainty as the expected increase of cell viability based on additive effects of zeaxanthin and α -tocopherol.

Effects of combinations of zeaxanthin with ascorbate on cytotoxicity induced by rose bengal and green light

During 20 min irradiation 0.35 mM ascorbate alone exerted a greater protective effect than zeaxanthin alone ($P < 0.001$) (Fig. 6a). Cell viability after exposure to RB and light in the presence of 0.7 mM ascorbate alone was similar to that in the presence of zeaxanthin alone (Fig. 6b).

Combinations of zeaxanthin with 0.35 or 0.70 mM vitamin C during 20 min irradiation significantly increased cell viability from ~42% to ~70% and ~54%, respectively ($P < 0.001$ for both concentrations of ascorbate) (Fig. 6a,b). A combination of 0.35 mM ascorbate with zeaxanthin during 20 min irradiation exerted a significantly greater protection than zeaxanthin alone ($P < 0.001$) (Fig. 6a). Combination of 0.7 mM ascorbate with zeaxanthin exerted a significantly smaller protection than zeaxanthin alone ($P = 0.002$) (Fig. 6b). Combination of 1.4 mM ascorbate with zeaxanthin exerted a considerably smaller protection than zeaxanthin alone ($P < 0.001$) (Fig. 6c). The increase in phototoxicity observed in the presence of 1.4 mM ascorbate and zeaxanthin was similar to that observed in the presence of 1.4 mM ascorbate alone. None of the ascorbate concentrations tested made significant differences between cells treated with ascorbate alone or in combination with zeaxanthin (Fig. 6).

Combination of any of the three concentrations of ascorbate with zeaxanthin during 45 min irradiation exerted a considerable increase in toxicity in comparison to cells without antioxidants or in the presence of zeaxanthin alone ($P<0.001$) (Fig. 6). The phototoxicity in the presence of zeaxanthin and ascorbate was similar for all three concentrations of ascorbate, and it was similar to that in the presence of ascorbate alone.

Effects of combinations of α -tocopherol with ascorbate on cytotoxicity induced by rose bengal and green light

During 20 min irradiation, 0.35 mM ascorbate alone exerted a similar protective effect as α -tocopherol alone (Fig. 7a). Cell viabilities after exposure to RB and light in the presence of higher concentrations of ascorbate were significantly smaller than in the presence of α -tocopherol ($P<0.001$) (Fig. 7b,c).

Combinations of α -tocopherol with 0.35 and 0.70 mM vitamin C during 20 min irradiation significantly increased cell viability from ~42% to ~86% and ~60%, respectively ($P<0.001$ for both concentrations of ascorbate) (Fig. 7a,b). For 0.35 mM ascorbate and 20 min irradiation there were significant increases in cell viabilities for cells treated with ascorbate in combination with α -tocopherol in comparison with cells treated only with ascorbate or only α -tocopherol ($P<0.001$) (Fig. 7a). There was no significant difference between cells treated with 0.7 mM or 1.4 mM ascorbate alone and cells treated with these concentrations of ascorbate in combination with vitamin E (Fig. 7b, c). Combination of 0.7 mM or 1.4 mM ascorbate with α -tocopherol exerted a significantly smaller protection than α -tocopherol alone ($P<0.001$) (Fig. 7b,c).

Combination of any of the three concentrations of ascorbate with α -tocopherol during 45 min irradiation exerted a considerable increase in toxicity in comparison to cells without antioxidants or in the presence of α -tocopherol alone ($P<0.001$) (Fig. 7). The phototoxicity in the presence of combinations of α -tocopherol and ascorbate was similar for all three concentrations of ascorbate and was comparable to the phototoxicity in the presence of ascorbate alone.

Effects of combinations of zeaxanthin and tocopherol with ascorbate on cytotoxicity induced by rose bengal and green light

A combination of zeaxanthin and α -tocopherol exerted significantly better protection than ascorbate alone during 20 min irradiation ($P=0.029$ for a comparison of 0.35 mM ascorbate alone with a combination of zeaxanthin and α -tocopherol where cell viability was increased from ~70% to ~75%; $P<0.001$ for both comparisons of a combination of zeaxanthin and α -tocopherol with 0.7 mM or 1.4 mM ascorbate alone where cell viabilities decreased from ~75% to ~56% and 5%, respectively) (Fig. 8).

A combination α -tocopherol, zeaxanthin and 0.35 mM ascorbate exerted the most effective protection by increasing cell viability to ~91% when compared with ~42% in the absence of antioxidants ($P<0.001$) (Fig. 8a). This increase was also significant in comparison to cells treated with only zeaxanthin or α -tocopherol ($P<0.001$).

A combination of 0.7 mM ascorbate, α -tocopherol, and zeaxanthin increased cell viability to ~65% which was a significant increase in comparison with 0.7 mM ascorbate alone ($P<0.001$) but the effect was significantly smaller than for a combination of zeaxanthin and α -tocopherol without ascorbate ($P<0.001$) (Fig. 8b). Cell viabilities after exposure to RB and light in the presence of 1.4 mM ascorbate in combination with zeaxanthin and α -tocopherol were similar as in the presence of 1.4 mM ascorbate alone (Fig. 8c).

Combination of any of the three concentrations of ascorbate with zeaxanthin and α -tocopherol during 45 min irradiation exerted a considerable increase in toxicity in comparison to cells without antioxidants or in the presence of α -tocopherol alone, zeaxanthin alone or a combination of both lipophilic antioxidants ($P < 0.001$ for each of these cases) (Fig. 8). The phototoxicity in the presence of combinations of α -tocopherol, zeaxanthin and ascorbate was similar for all three concentrations of ascorbate and it was similar to that in the presence of ascorbate alone.

For cells cultured in MEM, ascorbate in combinations with zeaxanthin, α -tocopherol or both lipophilic antioxidants did not significantly affect phototoxic effects of RB in comparison to cells treated with lipophilic antioxidants without ascorbate (Supplemental Fig. S4).

DISCUSSION

Protective and deleterious effects of ascorbate on ARPE-19 cells during photooxidative stress

To model the RPE *in vivo* in the ageing retina and particularly in the retina suffering from AMD where there is substantial accumulation of weakly chelated iron, we cultured ARPE-19 cells in iron-rich medium DMEM/F12. Our results demonstrate that ascorbate can offer substantial protection against photooxidative damage to these cells but the protective effect is limited to a small range of concentrations of vitamin C (0.35–0.70 mM) and short exposure time to photooxidative stress. The protective effects of vitamin C are likely due to its ability to scavenge singlet oxygen which is the predominant reactive species generated by RB (44). The small concentrations of vitamin C which offer protection at a short irradiation time of 20 min, exacerbate the photocytotoxicity at a longer irradiation time of 45 min. The increase in phototoxicity in the presence of ascorbate can be due to interactions of ascorbate (AscH^-) with photoexcited photosensitizer in its triplet state (^3RB) and/or with singlet oxygen ($^1\text{O}_2$) leading to increased production of free radicals and hydrogen peroxide, respectively (10, 17, 18, 42, 53):

Moreover, ascorbate can enhance photodynamic damage by redox cycling of traces of iron ions (10, 19–25). Ascorbate can reduce Fe^{3+} either directly:

or *via* generation of the reduced form of RB, $\text{RB}^{\bullet-}$ (as shown in Eq. 3) which, in turn, reduces Fe^{3+} (58):

Reduced iron can be oxidized by hydrogen peroxide (formed, for example, by reaction depicted in Eq. 4) leading to production of the most reactive free radical produced in cells, the hydroxyl radical, $\bullet\text{OH}$:

The interaction of hydroxyl radical with unsaturated lipids is likely to lead to a free radical chain of lipid peroxidation (59). Each lipid hydroperoxide formed as a result of lipid peroxidation can interact with iron ions giving rise to a new chain of lipid peroxidation, propagating rapidly the oxidative damage.

It appears that quenching of singlet oxygen by ascorbate (Eq. 4) is protective only for a short period of time whereas during an extended exposure, the prooxidant actions (Eqs. 3–7) dominate. The importance of the reactions responsible for redox-cycling of iron ions in enhancing phototoxicity mediated by ascorbate is underlined by the results of comparison of cells cultured in DMEM/F12 and in MEM. For cells cultured in MEM, ascorbate did not exert any deleterious effects even at the highest concentration tested of 2 mM.

Intriguingly, for cells cultured in MEM, ascorbate did not exert any significant protection against photooxidative damage. It can be expected that due to quenching of singlet oxygen by ascorbate, hydrogen peroxide is generated. Thus, the mechanism of damage may shift from singlet oxygen-mediated in the absence of ascorbate towards H_2O_2 -mediated in the presence of ascorbate. Cells cultured under conditions of low iron seem to be almost equally susceptible to damage whether it is induced by singlet oxygen or hydrogen peroxide, whereas this is no longer true for cells cultured in the presence of high iron. It can be expected that cells cultured in the presence of supplemented iron ions may have up-regulated antioxidant defences, such as catalase so they may be better equipped to decompose H_2O_2 and this can explain the protective effect of ascorbate where cells are more susceptible to damage by singlet oxygen than by hydrogen peroxide. Ascorbate can redox cycle iron ions and therefore abundant iron ions are expected to facilitate production of free radicals initiating a chain of peroxidation and therefore amplifying the damage. Therefore, at higher concentrations of ascorbate and/or longer irradiation times, the prooxidant effects of ascorbate can be seen.

It may be suggested that the protective and deleterious effects of ascorbate could be in part due to oxidation of ascorbate, yielding dehydroascorbate. In contrast to ascorbate, the dehydroascorbate can pass through glucose channels in the plasma membrane (60). Once inside the cell, dehydroascorbate can be reduced to ascorbate and therefore enhance the intracellular antioxidant protection. The reduction can be either enzymatic and dependent on glutathione or NADPH or non-enzymatic (where glutathione or homocysteine is involved). It can be suggested that cells cultured in media with high iron content have up-regulated antioxidants, including those with ascorbate reductase activity, and therefore an increased rate of generation of intracellular ascorbate. Intracellular ascorbate in cells rich in iron can have deleterious consequences because ascorbate can release iron from ferritin (61). The released iron can be redox-cycled by ascorbate and hydrogen peroxide resulting in increased production of reactive oxygen species. Cells cultured in media without supplemental iron are less likely to accumulate significant amounts of ferritin-bound iron so the presence of ascorbate is not so damaging as in case of cells cultured in Fe-supplemented media.

It needs to be stressed that concentrations of ascorbate used in our experiments were physiologically relevant. Under physiological conditions RPE cells can be exposed to up to 0.25 mM ascorbate on their basal side where they can be exposed to the blood plasma after a

saturating oral dose of ascorbate (62). In brain tissues, intracellular concentrations of ascorbate reach ~10 mM in neurons and ~1 mM in glia (16).

Effects of zeaxanthin, tocopherol and their combinations on RB-mediated phototoxicity in the absence and presence of ascorbate

Both lipophilic antioxidants, zeaxanthin and α -tocopherol offered partial protection from RB-mediated phototoxicity which is consistent with our previous studies on liposomes and ARPE-19 cells demonstrating effective quenching of singlet oxygen and scavenging of free radicals by zeaxanthin and α -tocopherol, respectively (9, 10).

Protective effects of combination of zeaxanthin and α -tocopherol are less than additive for 20 min and 45 min irradiation times for cells cultured in DMEM/F12 whereas at 90 min appear synergistic, mainly because zeaxanthin alone does not exert significant protection for this exposure time. Protective effects of combination of zeaxanthin and α -tocopherol are additive for cells cultured in MEM.

Zeaxanthin alone does not significantly influence the effects of ascorbate on phototoxicity to ARPE-19 cells. In contrast, α -tocopherol can substantially increase viabilities of cells treated with 0.35 mM ascorbate, RB and light for 20 min. The best protective effect can be achieved by a combination of all three antioxidants but is observed only for 0.35 mM ascorbate and 20 min irradiation time for cells cultured in DMEM/F12.

The enhanced protection by a combination of three antioxidants may be ascribed to protection of the critical antioxidant by other antioxidants (2). In this case, zeaxanthin may be viewed as the critical antioxidant which is consumed rapidly unless protected by α -tocopherol or ascorbate. Zeaxanthin can play a role as an antioxidant by quenching singlet oxygen and/or scavenging free radicals. Quenching of singlet oxygen by zeaxanthin is close to the diffusion controlled limits and occurs mostly by energy transfer from the electronically excited state of oxygen followed by thermal deactivation of zeaxanthin so less than 1/1000 of zeaxanthin can be chemically modified by singlet oxygen (2, 63). In contrast, interaction of zeaxanthin with free radicals leads to electron/hydrogen transfer or addition of zeaxanthin to the free radical and formation of a free radical adduct.

As a result of electron donation by zeaxanthin, a free radical cation of zeaxanthin is formed. Both α -tocopherol and ascorbate can reduce the semi-oxidized form of zeaxanthin and therefore repair the original molecule (2). Moreover, α -tocopherol or ascorbate can inhibit zeaxanthin degradation simply by scavenging free radicals and therefore preventing from their interactions with zeaxanthin. Repair of semi-oxidized α -tocopherol by vitamin C can also lead to enhanced protection offered by these two antioxidants, one of which - α -tocopherol is lipophilic and therefore limited with its action to the lipid membrane unlike the hydrophilic ascorbate which can act as antioxidant in the extracellular fluid, the cytoplasm and at the interface of the aqueous compartment and the lipid membrane (64).

Increasing concentrations of ascorbate or extending the irradiation time diminishes the protective effect of lipophilic antioxidants, leading eventually to exacerbated phototoxicity similar to that observed in the presence of ascorbate without any added lipophilic antioxidant. These results suggest that there is a very narrow range of concentrations of ascorbate which can exert beneficial effects within a short time of increased photooxidative stress imposed on RPE cells. By increasing the length of exposure to photooxidative stress the beneficial effects of ascorbate are replaced by exacerbated toxicity. The results of this *in vitro* study cannot be easily extrapolated to the human retina *in vivo*. Firstly, ARPE-19 cells have been shown to exhibit several, but not all, functional and structural properties of human RPE cells (65). Secondly, the mechanism of damage induced in our experiments by RB and

light may be different that the potential oxidative damage that can occur in the RPE cells in the retina *in vivo*.

Nevertheless, an adequate antioxidant protection of the RPE is of vital importance. Zeaxanthin together with vitamin E and ascorbate are present in the RPE-choroid complex and in adjacent photoreceptor outer segments (2). Dysfunction and death of the RPE is suggested to be the primary site of damage leading to the development of age-related macular degeneration (AMD). The effect of antioxidant supplements on development and progression of AMD was tested in a large multicentre clinical trial, Age-Related Eye Disease Study (66). It has been demonstrated that antioxidant mixture of vitamin C, vitamin E and optional β -carotene taken daily together with zinc for a period of ~5 years slowed down by 25% the progression of AMD from a moderate to an advanced form. Vitamin C, vitamin E and zeaxanthin are some of the compounds currently tested in AREDS II aimed to evaluate their efficacy against progression of AMD (<http://clinicaltrials.gov/ct2/show/NCT00345176>) and the results of this clinical trial are expected in 2013. The total intake of vitamin C from AREDS supplement (500 mg) and Centrum Silver (60 mg), even if taken together, are expected to raise the plasma levels of ascorbate to not more than 100 μ M (56), a concentration at least 3.5-fold lower than the concentrations enhancing phototoxicity in our experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ARPE-19 cell morphology 17 h after 1 h exposure to the indicated concentrations of rose bengal and green light.

Figure 2.

Absorbance of formazan formed by control cells (without RB) kept in dark or exposed for 1 h to light during MTT assay performed at indicated times post-exposure to light (a). Reductive activity of ARPE-19 cells as a function of rose bengal concentration and the time between the MTT assay and the end of incubation in dark (D) or exposure to visible light (L) (b). Bars/symbols represent means while error bars – SEMs.

Figure 3.

Rose bengal (RB) dose- and exposure time -dependence on reductive activity of ARPE-19 cells measured 1 h after completing exposure to RB and green light. Symbols represent means while error bars – SDs.

Figure 4.

Effects of indicated concentrations of ascorbate on reductive activity of ARPE-19 cells after exposure to visible light in the presence and absence of rose bengal (RB). Prior to the exposure cells were cultured for at least three passages in iron-rich DMEM/F12. Symbols represent means while error bars – SDs.

Figure 5.

Effects of zeaxanthin, vitamin E and their combination on reductive activity of ARPE-19 cells after exposure to visible light in the presence and absence of rose bengal (RB). Prior to the exposure cells were cultured for at least three passages in iron-rich DMEM/F12. Symbols represent means while error bars – SDs.

Figure 6.

Effects of indicated concentrations of vitamin C in combination with zeaxanthin on reductive activity of ARPE-19 cells after indicated times of exposure to light in the presence and absence of rose bengal (RB). Prior to the exposure cells were cultured for at least three passages in iron-rich DMEM/F12. Symbols represent means while error bars – SDs.

Figure 7.

Effects of indicated concentrations of vitamin C in combination with vitamin E on reductive activity of ARPE-19 cells after indicated times of exposure to light in the presence and absence of rose bengal (RB). Prior to the exposure cells were cultured for at least three passages in iron-rich DMEM/F12. Symbols represent means while error bars – SDs.

Figure 8.

Effects of indicated concentrations of vitamin C in combination with zeaxanthin and vitamin E on reductive activity of ARPE-19 cells after indicated times of exposure to light in the presence and absence of rose bengal (RB). Prior to the exposure cells were cultured for at least three passages in iron-rich DMEM/F12. Symbols represent means while error bars – SDs.