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Studies on the Singlet Oxygen Scavenging Mechanism of Human Macular Pigment

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Abstract

It is thought that direct quenching of singlet oxygen and scavenging free radicals by macular pigment carotenoids is a major mechanism for their beneficial effects against light-induced oxidative stress. Corresponding data from human tissue remains unavailable, however. In the studies reported here, electron paramagnetic resonance (EPR) spectroscopy was used to measure light-induced singlet oxygen generation in postmortem human macula and retinal pigment epithelium/choroid (RPE/choroid). Under white-light illumination, production of singlet oxygen was detected in RPE/choroid but not in macular tissue, and we show that exogenously added macular carotenoids can quench RPE/choroid singlet oxygen. When the singlet oxygen quenching ability of the macular carotenoids was investigated in solution, it was shown that a mixture of *meso*-zeaxanthin, zeaxanthin, and lutein in a ratio of 1:1:1 can quench more singlet oxygen than the individual carotenoids at the same total concentration.

Keywords

Human macula; singlet oxygen; electron paramagnetic resonance (EPR)

1. Introduction

Macular pigment (MP) was first described as a “yellow spot” centered on the fovea of the human eye in the eighteenth century, and it was classified spectroscopically as a xanthophyll carotenoid by Wald in 1945 [1], but it was not until 1985 that Bone and Landrum chemically identified that the macular pigment is a mixture of the carotenoids lutein and zeaxanthin [2]. Macular pigment is diffusely found in the peripheral retina, but it is highly concentrated (~100-fold) in the foveal region of the macula, often exceeding a peak value of 1 mM in many humans [3–7]. In addition to spatial specificity, there is also remarkable chemical specificity of uptake into the human macula. Despite over a dozen readily detectable carotenoids found in human serum, only lutein, zeaxanthin, and their metabolites are found in the retina. In the fovea, the ratio of (3R,3’R,6’R)-lutein to (3R,3’R)-zeaxanthin to (3R,3’S-*meso*)-zeaxanthin is 1:1:1, while in the peripheral retina, lutein predominates over the zeaxanthins by a 3:1:0 ratio [1,6, 8].

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In nature, carotenoids account for the red, orange, and yellow colors of fruits and vegetables, as well as animal skin and other external features [9–11]. The role of carotenoids in photosynthesis has been extensively investigated, showing that their two major functions are light harvesting and photoprotection [12,13]. Their potential function as stabilizers of biological membrane structure has also been reported [14]. In addition, some studies have shown that these compounds may help reduce the risk of certain types of cancer and prevent heart disease and stroke [15].

The biological role of macular pigment carotenoids is not definitively known yet. According to their known functions and chemical properties, two major potential roles have been proposed: (1) enhancement of visual acuity and (2) protection against light-induced oxidative damage, particularly with respect to age-related macular degeneration (AMD) [1,16–20]. High carotenoid intake and serum levels are associated with a 43% risk reduction for exudative AMD [21]. In support for this mechanism, Thomson *et al* reported that when quail exposed to bright light were fed zeaxanthin-supplemented diets, they had significantly less light-induced photoreceptor apoptosis than those fed with carotenoid-deficient diets [22]. Moreover, several oxidation products of lutein and zeaxanthin are enriched in human retina, further supporting the antioxidant ability of MP [23]. When Bhosale *et al* purified a zeaxanthin-binding protein from the macula of human eye [24], they demonstrated that zeaxanthin when bound to its binding protein shows a higher ability to protect model lipid membranes from oxidation [25]. Meanwhile, *in vitro* experiments have shown that carotenoids can scavenge most reactive oxygen species [26,27] and that retinal, a component of photoreceptor pigment in retina, is a source of singlet oxygen [28]. Two possible mechanisms for how the macular pigment performs its protective function have been suggested: (1) filtering blue light to prevent the formation of reactive oxygen species, especially singlet oxygen in retina, or (2) reducing oxidation by directly quenching singlet oxygen and related free radicals in the retina [29,30]; however, there is still no direct evidence to definitively support either mechanism, and it remains unclear if singlet oxygen can even be generated in ocular tissues. In this work, singlet oxygen generated in human macula and retinal pigment epithelium/choroid (RPE/choroid) was measured by electron paramagnetic resonance (EPR) spectroscopy using a spin-trapping agent, and the current status of the preventive mechanisms of MP is discussed.

2. Materials and Methods

2.1 Reagents

Benzene, 2, 2, 6, 6-tetramethyl-4-piperidone (TEMP), and toluidine blue (TB) were purchased from Sigma (St. Louis, MO). HPLC-grade methylene chloride, methanol, and hexane were ordered from Fisher Scientific (Pittsburgh, PA).

2.2 Preparation of macular and retinal samples

Human donor eyes (approximately twenty eyes from donors 40–50 years old; equal representation of males and females) were obtained from the Utah Lions Eye Bank. Tissue procurement and distribution complied with the tenets of the Declaration of Helsinki. Dissection of the globes was performed on ice under dim light. Macular RPE/choroid and retina were excised with an 8-mm circular trephine, and vitreous was removed by a pipette during the dissection. To extract the MP carotenoids from macula, two maculae were homogenized in 500 μl of benzene on ice by a probe sonicator, and then the sample preparation was transferred into an ice water tub for a 30-min bath sonication. Finally, the sample preparation was centrifuged at 10000 × *g* for 5 min. The supernatant was collected for MP carotenoid analysis by HPLC, and the pellet, referred to as “carotenoid-depleted macular tissue”, was used for EPR spectroscopy.

2.3 HPLC

The HPLC equipment (Thermo Separation Products Inc., San Jose, CA) had an autosampler, a two channel solvent degasser, a binary gradient pump, and a UV-visible photo-diode array detector. HPLC grade solvents from Fisher Scientific (Pittsburgh, PA) were used as the mobile phases. The macular carotenoids extracted from macula were dissolved in HPLC mobile phase [hexane: dichloromethane: methanol: N, N'-diisopropylethylamine (80:19.2:0.7:0.1 v/v)]. HPLC separation was carried out at a flow rate of 1.0 ml per min on a cyano column (Microsorb 250 mm length × 4.6 mm i.d., Varian Inc., Palo Alto, CA). The columns were maintained at room temperature, and the HPLC detector was operated at 450 nm. Peak identities were confirmed by photo-diode array spectra and by co-elution with authentic standards as necessary.

2.4 EPR detection of singlet oxygen

Electron paramagnetic resonance (EPR) spectroscopy is employed to detect the formation of singlet oxygen as described in previous reports [31–33]. In brief, singlet oxygen can oxidize TEMP (a sterically hindered amine) to a stable nitroxide radical, TEMPO, which can be detected by EPR spectroscopy [34]. Macula, carotenoid-depleted macular tissue, and foveal RPE/choroid were incubated with 1 ml of 80 mM TEMP solution (in ethanol) for 5 min. Ethanol was removed either by centrifugation or by blotting, and the tissues were put into EPR cells. The EPR cells were sealed after an argon gas purge and then exposed to light for 5 min at room temperature (22 °C). The intensity of the light reaching the sample was around 3000 lux after passing through a saturated $CuSO_4$ solution (5 cm light path). EPR spectra were immediately recorded with a Bruker EMX spectrometer at room temperature (22 °C) using the following instrument settings: microwave power, 49.9 mW; modulation amplitude, 0.23 G; modulation frequency, 100 KHz; time constant, 40.9 ms; receiver gain, 1.0×10^5.

2.5 DMF assay

The ability of carotenoids to provide photoprotection against singlet oxygen was measured using a previously published method [28]. Briefly, 1 ml of the photosensitizer 5 mM toluidine blue (TB) in ethanol was illuminated (as in the EPR experiments) for 5 min in the presence of 0.2 mM 2, 5-dimethylfuran (DMF). DMF is very reactive with singlet oxygen, and its rapid photo-oxidation is evidenced by disappearance of its 215 nm absorption peak recorded on a Thermo Smart 3000 spectrophotometer. When 25 ul of 2 mM carotenoid stock solutions are added to 1 ml of this DMF reaction solution (final concentration of carotenoid = 50 μM), the DMF photo-oxidation rate is reduced. The percent inhibition of light-induced photo-oxidation of DMF by added carotenoids was then recorded for each carotenoid tested.

3. Results

3.1 Preparation and characterization of carotenoid-depleted macular tissue

To investigate if MP has the function of quenching singlet oxygen, experiments were designed to measure the production of singlet oxygen in macula and in carotenoid-depleted macular tissue (the remnant portion of macular tissue after MP has been extracted). Tetrahydrofuran (THF) was initially used to extract the MP from human macula for the preparation of carotenoid-depleted macular tissue since it has been used in similar prior studies [35–37]. Fig. 1a shows the HPLC chromatogram of pigment extracted from human macula by THF. According to their absorption spectra and chromatographic characteristics, three major peaks eluted at 3.4 min, 9.8 min and 10.9 min, and they were identified as retinal, lutein, and zeaxanthin, respectively. As previous *in vitro* experiments have demonstrated that retinal molecules are probably the major photosensitizers located in retina required for photodynamic reactions [28], we decided to modify the MP extraction protocol to keep the retinal in the

macular residue. To ensure retinal and as many other components other than MP can be left in the residue, benzene was selected to replace THF because it has often been used to extract carotenoids selectively from many biological systems to study the function of carotenoids in pigment-protein complexes [38–40]. Fig. 1b shows the chromatogram of MP extracted by benzene with the modified protocol. Compared to the elution peaks of compounds extracted by THF (Fig. 1a), peak 1 (retinal) in Fig. 1b was absent, and the small peaks nearby, most likely various lipids and/or proteins, became less intense. The retention time and absorption spectrum of the retinal standard in Fig. 1c matches very well with that of peak 1 shown in Fig. 1a. Meanwhile, about 80% of lutein and zeaxanthin were still extracted from the macula by benzene (Fig. 1b). This carotenoid-depleted macular tissue was used for further studies.

3.2 Singlet oxygen measurement in human macula by EPR

Fig. 2a and Fig. 2d show the EPR detection of singlet oxygen in 50 μl of 1mM toluidine blue (a prototypic photosensitizer) solution in the dark and after exposure to light for 5 min. The signal in Fig. 2d is a typical spectrum for TEMPO, the singlet oxygen adduct with TEMP. No EPR signal was detected in macula or carotenoid-depleted macular tissue under light or dark conditions (Fig. 2b, 2c, 2e, 2f), indicating there is no formation of singlet oxygen in these tissues. The rate of singlet oxygen formation of toluidine blue is very close to that of retinal under our experimental conditions [31]. According to the rhodopsin content [41], the concentration of retinal in human retina is calculated to be 10–30 mM. This means that if MP is able to directly quench singlet oxygen formed in macula, singlet oxygen should be detectable in carotenoid-depleted macular tissue after light treatment as the quantity of retinal and, presumably, singlet oxygen is around 30 times as much as that found in a 1 mM toluidine blue solution.

3.3 Singlet oxygen measurement in human RPE/choroid by EPR

Lipofuscin is thought to be a photosensitizer of the RPE/choroid [1,42,43]. Therefore, we also tested if singlet oxygen can be generated in RPE/choroid. EPR spectroscopy was carried out on subfoveal RPE/choroid in dark and light. An EPR signal was present in subfoveal RPE/choroid even in the dark (Fig. 3a), but it is not a spectrum consistent with TEMPO, and it most likely represents melanin radical which is a semiquinone species [44,45]. When treated with light, there were two new small peaks on each side of this melanin-radical signal (Fig. 3d). Their positions are very close to that of TEMPO, and their line widths are around 2.6 G (Fig. 2d). When subfoveal RPE/choroid was incubated with macular tissue or zeaxanthin, these two small peaks disappeared, but the melanin-radical signal was still there (Fig. 3b, 3c, 3e, 3f), suggesting that MP can quench light-induced singlet oxygen. To test whether this is truly a signal from TEMPO, the difference spectrum of foveal RPE/choroid in light and dark was obtained by subtracting Fig. 3a from Fig. 3d. The intensity of the middle peak in the difference spectrum is stronger than that of a standard spectrum of TEMPO, and its width is wider than expected for TEMPO (Fig. 4). Most likely, this distorted EPR spectrum is due in part to light-generated melanin radicals because Seagle *et al* have shown that high intensity light can increase the melanin radical signal of RPE culture [45]. The difference spectrum, therefore, looks like overlapping spectra between melanin radical and TEMPO especially since the locations of the three lines at 3448.6 G, 3464.5 G and 3480.4 G were very similar to the spectrum of TEMPO (3448.5 G, 3464.0 G and 3479.5 G) (Fig. 2d). These results suggest that singlet oxygen can be formed in RPE/choroid and that MP can quench them.

3.4 The ability of macular carotenoid to quench singlet oxygen

The quenching abilities of *meso*-zeaxanthin, zeaxanthin, and lutein were investigated by UV-absorption spectroscopy using DMF as the reporter compound. The order of the ability of MP to quench singlet oxygen was *meso*-zeaxanthin >zeaxanthin >lutein. As the ratio of *meso*-

zeaxanthin, zeaxanthin and lutein is 1:1:1 in the center of human macula [3,18], we also tested an iso-molar mixture of these three carotenoids at a physiological ratio of 1:1:1. We found that this mixture had the strongest ability to quench singlet oxygen, quenching 1.37 times as much singlet oxygen as *meso*-zeaxanthin alone, 1.91 times as much as zeaxanthin alone, and 2.40 times as much as lutein alone (Fig. 5).

4. Discussion

Singlet oxygen can be produced by energy transfer from triplet states of sensitizers according to the following scheme [31]:

$$^{3}\text{Sensitizer} + ^{3}O_2 \rightarrow ^{1}\text{Sensitizer} + ^{1}O_2$$

Retinal has been thought to be the major photosensitizer for singlet oxygen formation in the human retina, but we did not detect singlet oxygen from macula or from carotenoid-depleted macular tissue even after intense light treatment (Fig. 2e and Fig. 2f), indicating that singlet oxygen probably cannot be generated by light in retina, especially since the light that reaches the retina is typically much weaker than the light we used in our experiments. On the other hand, our EPR data from RPE/choroid showed that singlet oxygen can be formed in illuminated human RPE/choroid, and it can be readily quenched by exogenous zeaxanthin. More importantly, similar singlet oxygen quenching can occur when the illuminated RPE/choroid tissue is incubated with human macular tissue. Since photoreceptor outer segment membranes are closely associated with retinal pigment epithelium (RPE) cells, this means that the low, but detectable [7], levels of MP in the outer retina may protect the highly polyunsaturated membranes of photoreceptor outer segments from the damaging effects of singlet oxygen produced by the RPE.

A DMF chemical assay was employed to investigate the singlet oxygen quenching ability of the macular carotenoids. The MP carotenoids, lutein, zeaxanthin and *meso*-zeaxanthin, are highly concentrated in the human fovea at a ratio of 1:1:1 [20]. Although the abilities of many carotenoids to quench singlet oxygen has already been well documented *in vitro* [27,28], data on *meso*-zeaxanthin and the mixture of these three carotenoids are not available yet. Zeaxanthin had a higher ability to quench singlet oxygen than lutein which is most likely due to its longer chain of conjugated double bonds, and the difference between *meso*-zeaxanthin and zeaxanthin is possibly is due to different interactions with the solvent environment. Our results also showed that a 1:1:1 mixture of the three carotenoids can quench singlet oxygen more efficiently than any of the three individually (Fig. 5). It is possible that when mixed at this physiological ratio, these three carotenoids may form specific aggregates, which could enhance their ability to quench singlet oxygen. A similar synergistic effect of zeaxanthin and its binding protein has been reported before [25]. This indicates that MP carotenoids composed in this ratio may optimally quench singlet oxygen and other reactive oxygen species in human retina, although *in vivo* data are still not available.

In biochemical systems, reactive oxygen species can be produced as by products of cellular metabolism or as products of photochemical reactions [46]. In this study, although our data showed that no singlet oxygen was detected in macula or carotenoid-depleted macular tissue under dark conditions (Fig. 2b and Fig. 2c), we still cannot completely rule out the presence of singlet oxygen generated through cellular metabolism in the retina because the EPR spectroscopy was performed on post-mortem human macular tissues. Cultured retinal cells could be employed to clarify this question. In addition, 20% of the carotenoids are still left in the carotenoid-depleted macular tissue, and these remaining carotenoids may be enough to quench singlet oxygen formed in the macular tissue. To exclude this possibility, carotenoid-

free macular tissue would need to be prepared. In this study, we measured only singlet oxygen in human ocular tissues; therefore, to completely understand the protective function of MP carotenoids, other reactive oxygen species should also be studied in human macular tissues in the future.

In conclusion, light can induce singlet oxygen formation in human RPE/choroid but not in human retina, and MP carotenoids located in photoreceptor outer segments may play a role to directly quench them; however, the majority of macular carotenoids is localized to the inner retina far away from the RPE layer. It is unlikely that these inner retinal carotenoids directly quench the singlet oxygen originating from the RPE, but they can still act as intrinsic filters of short wavelength light to prevent or reduce the generation of singlet oxygen in RPE in the first place.

Abbreviations

EPR	electron paramagnetic resonance
RPE	retinal pigment epithelium
AMD	age related macular degeneration
THF	tetrahydrofuran
DMF	2, 5-dimethylfuran
TEMP	2, 2, 6, 6-tetramethyl-4-piperidone
TB	toluidine blue
MP	macular pigment

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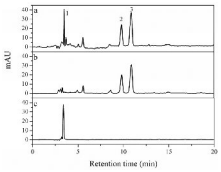

Fig. 1. HPLC chromatogram of pigments from macula

(a) Pigments extracted from macula by THF; (b) Pigments extracted from macula by benzene; (c) Standard retinal.

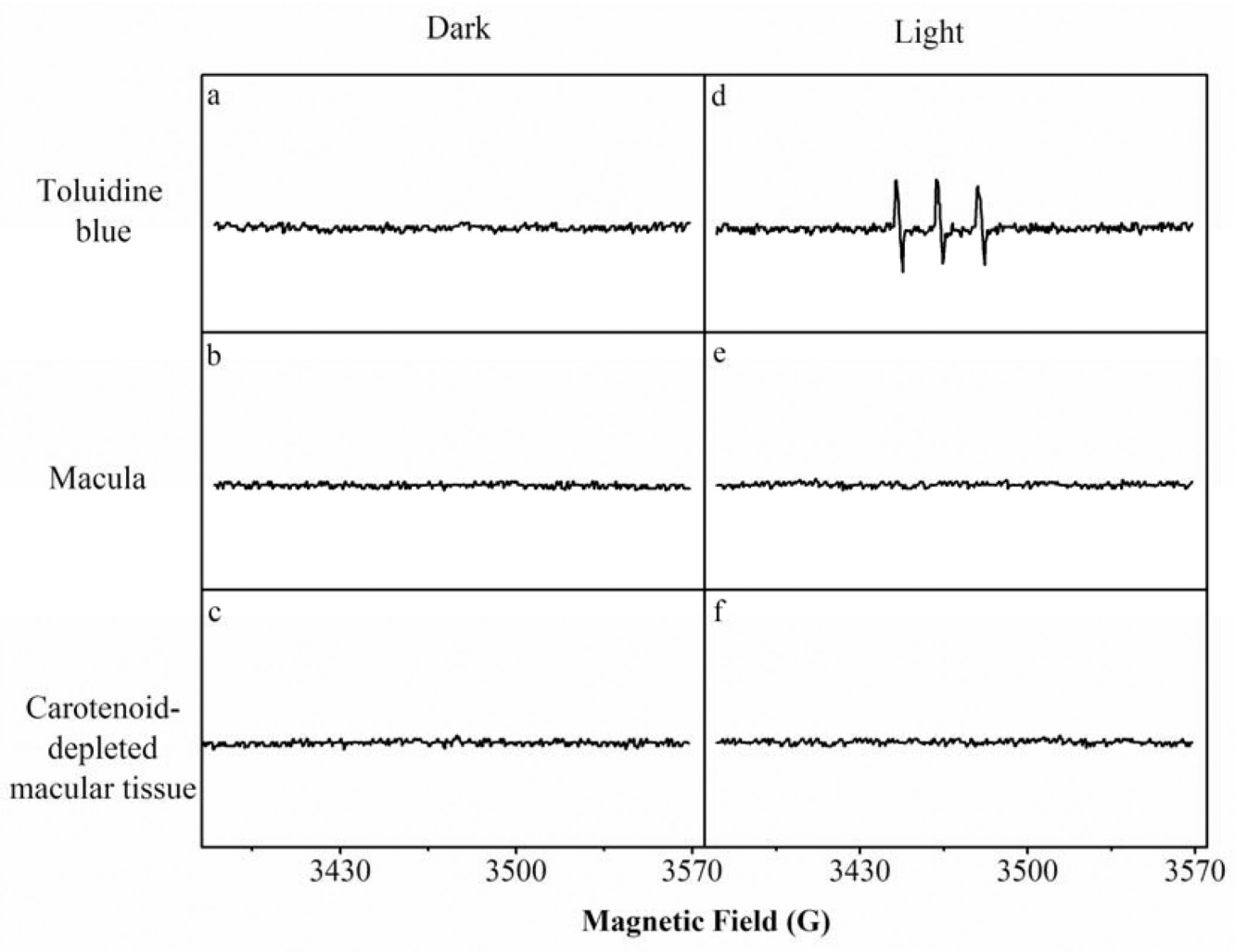

Fig. 2. EPR spectra of the singlet oxygen adduct TEMPO in macula

(a) Toluidine blue (1mM), (b) Macula, and (c) Carotenoid-depleted macular tissue incubated with 80 mM TEMP under dark conditions; (d) Toluidine blue (1 mM), (e) Macula, and (f) Carotenoid-depleted macular tissue incubated with 80 mM TEMP after 5-min of intense light treatment.

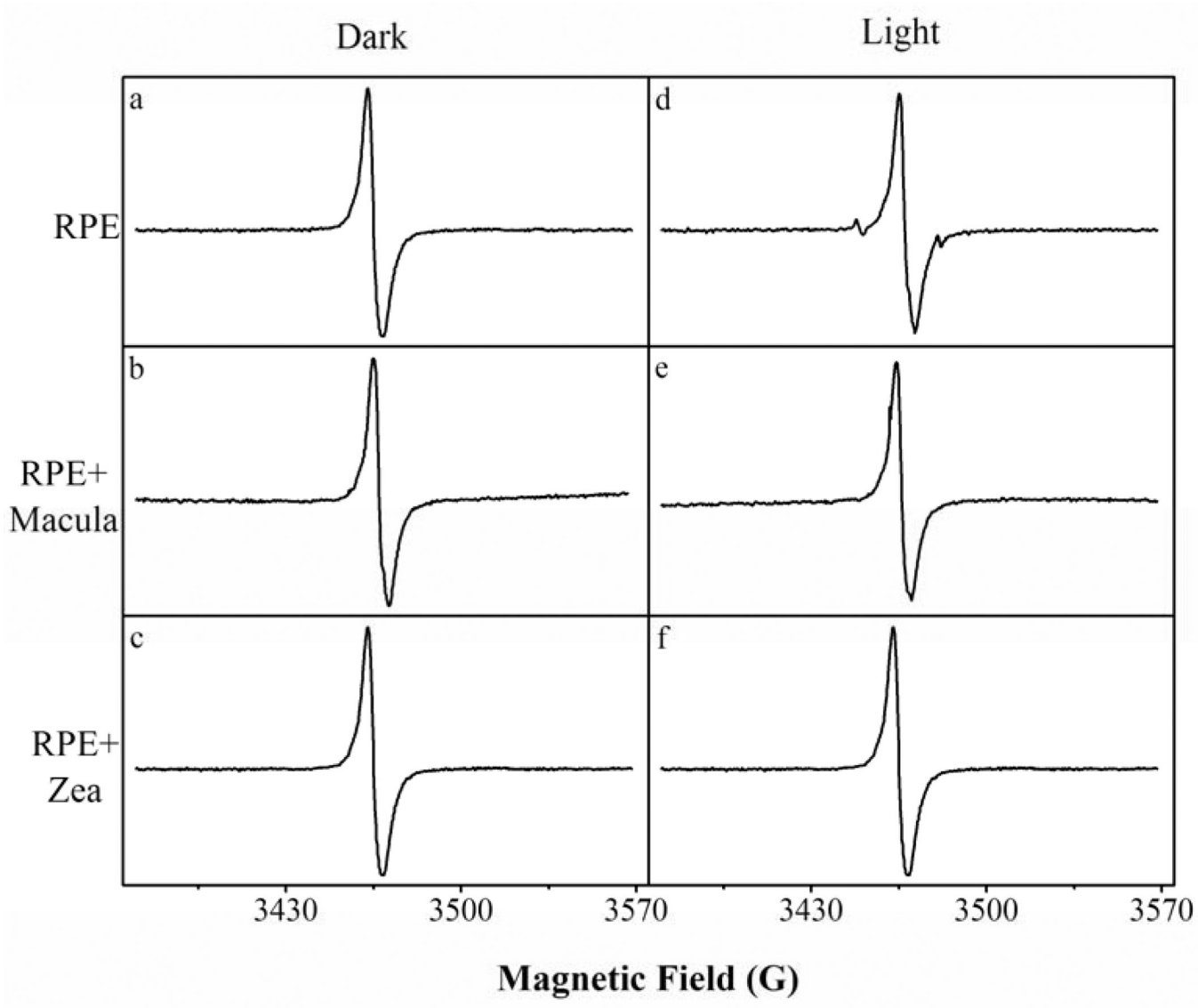

Fig. 3. EPR spectra of the singlet oxygen adduct TEMPO in foveal RPE

(a) Foveal RPE, (b) Foveal RPE and macula, and (c) Foveal RPE and 1 mM zeaxanthin incubated with 80mM TEMP under dark conditions; (d) Foveal RPE, (e) Foveal RPE and macula, and (f) Foveal RPE and 1 mM zeaxanthin incubated with 80 mM TEMP after 5-min light treatment.

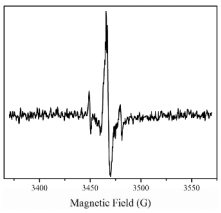

Fig. 4. Difference spectrum obtained by subtraction of Fig. 3a from Fig. 3d

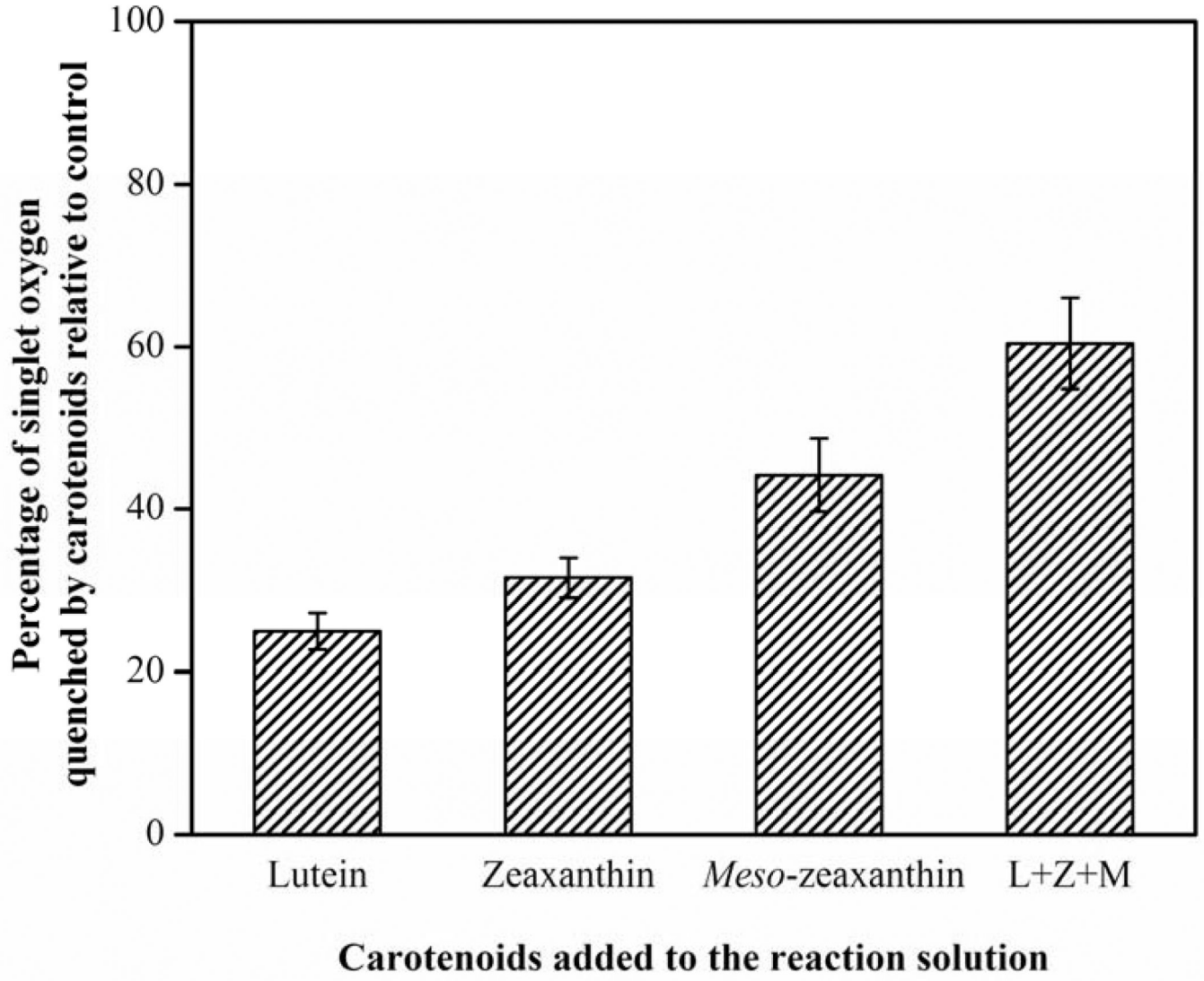

Fig. 5. Measurement of singlet oxygen quenching abilities of MP carotenoids by DMF assay

25 ul of each carotenoid (2 mM) stock solution was added to 1 ml of 0.2 mM DMF reaction solution in ethanol, and the absorption spectra at 215 nm were recorded on a spectrophotometer after the solution was illuminated for 5 min. The difference spectra between DMF and illuminated DMF solution was used as the control. The difference spectra between illuminated carotenoid solution and illuminated DMF solution was used to calculate the relative percentage of singlet oxygen quenched by each carotenoid.