



Retinal Light Damage: Protective Effect of α -Tocopherol

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Abstract: We studied histologically the protective effect of α -tocopherol to retinal light damage. After 3-week-old albino rats were fed with an α -tocopherol deficient or supplemented diet and kept in a 12-hour dim light/12-hour dark environment for 8 weeks, each animal was exposed to intense light (2500 lux) for 1, 3, 6, 12, 24, and 72 hours. The eyes were enucleated and prepared for transmission electron microscopy study and image analysis of phagosomes. Before light exposure, the α -tocopherol content of the neural retina of the deficient and supplemented groups was 0.3 μ g and 23.34 μ g, respectively. After 1- and 3-hour exposures, morphological changes in the retinal pigment epithelium and photoreceptor membranes were more extensive in the deficient group than in the supplemented group. After a 24-hour exposure, pyknotic photoreceptor nuclei were more numerous in the deficient group than the supplemented group. After 3-, 6-, and 12-hour exposures, large phagosomes were more numerous in the deficient group than in the supplemented group. These findings suggest that α -tocopherol can protect the retina from light injury for up to 24 hours of exposure. **Jpn J Ophthalmol 1997;41:160-167** © 1997 Japanese Ophthalmological Society

Key Words: α -Tocopherol, phagosome, retinal light damage, transmission electron microscopy.

Introduction

Polyunsaturated fatty acids are rich in photoreceptor membranes.^{1,2} One cause of retinal light damage is thought to be autoxidation of these membranes³ induced by free radicals that occur during light exposure.^{4,5} Photoreceptor membranes contain α -tocopherol,⁶ a scavenger of free radicals⁷ known to prevent retinal light damage.⁸ However, the precise significance of a α -tocopherol in retinal light protection is controversial.⁹⁻¹⁴ We previously reported no significant difference in electroretinogram amplitude and light microscopic findings between rats fed α -tocopherol supplemented or deficient diets.¹⁵ The present study used the same animals and light source¹⁵ and reinvestigated the effect of α -tocopherol using scanning electron microscopy and image analysis of phagosomes in the retinal pigment epithelium (RPE).

Materials and Methods

Animals and Diets

Weaned 3-week-old male albino rats (Wistar strain) were divided into two groups: one (SUP) was fed an α -tocopherol supplemented diet (AIN-76TM purified diet¹⁵ containing 58.5 mg/100 g tocopherol nicotinate). The other group (DEF) received an α -tocopherol deficient diet containing less than 0.1 mg/100 g whole tocopherol (both diets offered by Eisai). Levels of other nutrients in both diets were adequate¹⁶ and equal. Animals had free access to the diet and water. All animals were kept in 12-hour dim light (3-5 lux)/12-hour dark cycles for 8 weeks. Light exposure began at 8:00 AM. In this study, we followed the guidelines for animal experimentation of Hamamatsu University School of Medicine.

Measurement of Tocopherol Content

After 8 weeks, we sacrificed two rats in each group and enucleated the eyes for measurement of the neural retinal levels of tocopherol, using the Abe et al¹⁷ high-performance liquid chromatography (HPLC): tocopherols were extracted from 1.0 mL of neural

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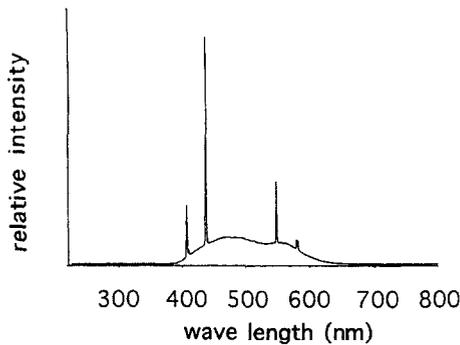


Figure 1. Spectrum of light source.

retinal homogenate with 5.0 mL of n-hexane, after addition of 2.0 mL of ethanol containing tocol as an internal standard. Five milliliters of the N-hexane extract was evaporated with N_2 gas at 40°C. The residue was dissolved in 500 μ L of n-hexane. Twenty milliliters of the solution was injected into the HPLC column (YMC GEL NH2-100-S3, 3 μ m internal diameter, 4.6 \times 50 mm, Yamamura Kagaku). The eluted tocopherols were determined separately with an F-1000 fluorescence spectrophotometer (exposure 290 nm, emitted 325 nm) (Hitachi).

Light Exposure

The remaining rats were exposed to intense light for 1, 3, 6, 12, 24, and 72 hours beginning at 8:00 AM. The light source consisted of three roentgen film viewers. Each viewer has three 15 W daylight fluorescent lights and a white plastic diffuser. They were placed on the top and both sides of a clear plastic cage containing the rats.

The spectrum of this light source is shown in Figure 1. Luminance was 2500 lux at the base of the cage and 2000 Candela/m². Room temperature was maintained at 20–22°C by air conditioning.

Histological Examination of the Retina

After exposure to the light, the animals were sacrificed by a lethal dose of 1.5–3.0 mL intraperitoneal xylazin. Three animals were used for each exposure time from each group. The eyes were immediately enucleated. After removing the anterior portion, the tissues were fixed in 2% glutaraldehyde solution in pH 7.4 phosphate buffer for 3 hours. Post-pole tissues were trimmed to 3 \times 3 mm and postfixed with 1% OsO₄ in phosphate buffer for 2 hours, then embedded in epoxy resin. Ultra-thin sections were cut with a diamond knife and stained with uranyl acetate and Sato's lead solution. These were examined

Table 1. Content of Tocopherol in Neural Retina Before Light Exposure

	Content of Tocopherol (μ g/g)		
	α -	β -	γ -
SUP	23.34	<0.40	<0.40
DEF	0.3	<0.25	<0.25

by transmission electron microscopy (JEM 100CX, JEOL). Similar sections were prepared from rats that were not exposed to intense light.

Phagosome Measurement

Sections of 1 μ m were taken from the same tissue blocks and stained with toluidin blue. These were photographed with slide film (Fujichrome Daylight 100) at a magnification of \times 1000. Phagosomes were defined as intensely stained inclusion bodies in cytoplasm and microvilli of RPE as described by LaVail.¹⁸ Fifteen micrographs (total range: 2700 μ m) from each regimen were projected in the slide viewer and areas of phagosomes were copied on tracing papers. The images of phagosomes were captured with an image scanner (Scan Jet IICx/T, Hewlett Packard) and analyzed with an NIH image on a Power Macintosh 7100/66AV.

LaVail defined the phagosomes greater than 0.75 μ m in any dimension (half the diameter of the rod outer segments) as "large phagosomes" in rat retina.¹⁸ Because the area of a circle with a diameter of 0.75 μ m is 0.44 μ m², we defined large phagosomes as those over 0.6 μ m².

Results

Tocopherol

Table 1 shows the tocopherol content of the neural retina of both groups prior to light exposure. The levels of α -tocopherol in the SUP group were 80 times greater than the DEF group.

Electron Microscopy

No abnormal findings were seen in the unexposed retina of both groups. After a 1-hour exposure, there was mitochondrial swelling in the RPE in both groups. The mitochondrial changes were more extensive in the DEF rats than in the SUP rats (Figures 2A and 2B). In the DEF group, the vacuolation of the RPE cytoplasm was also found (Figures 3A and 3B). The photoreceptor membranes of the DEF rats

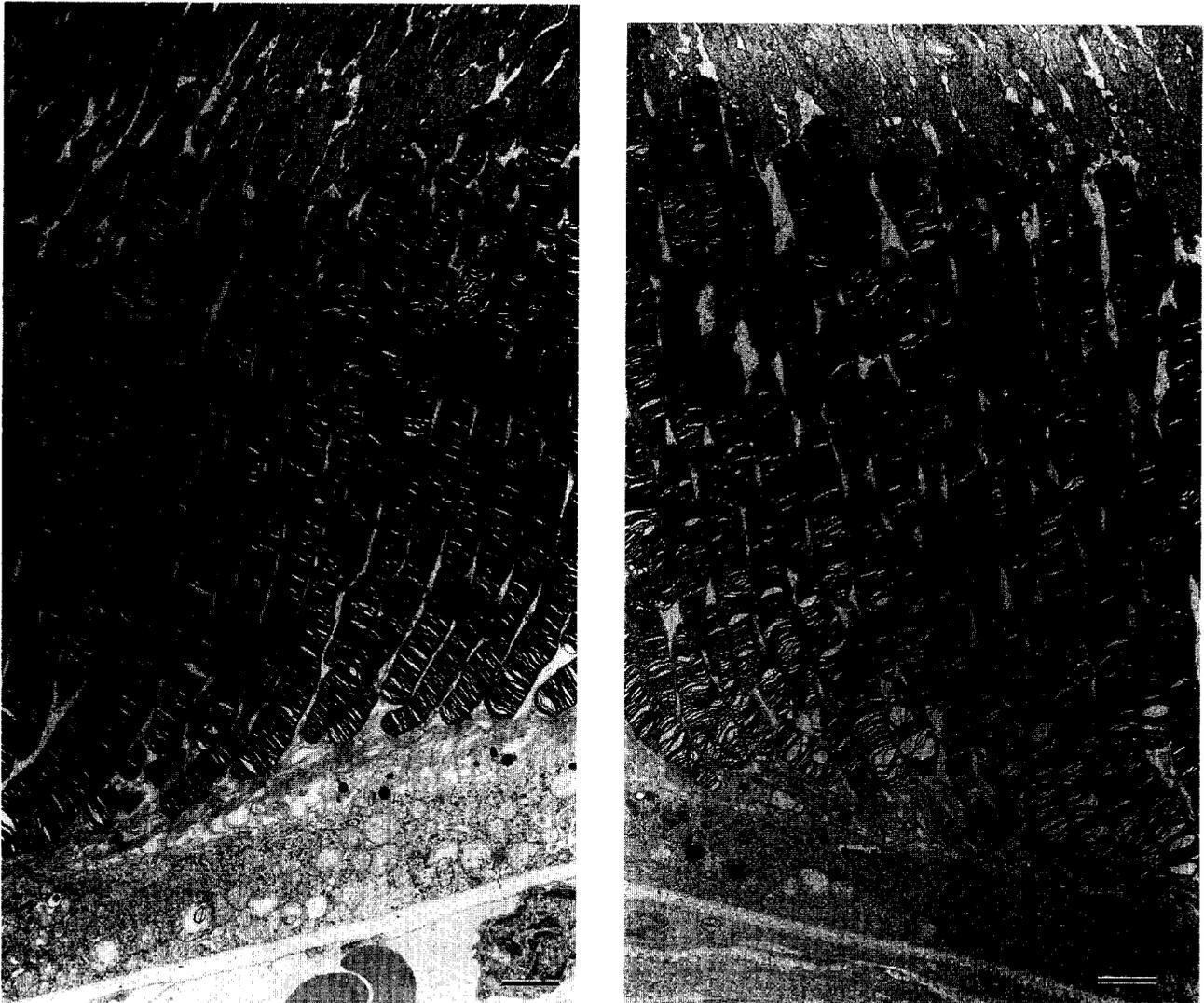


Figure 2. Electron micrographs of retina after 1-hour exposure. (A) SUP. (B) DEF. Mitochondrial swelling in RPE of both groups. Normal photoreceptor membranes in SUP (A). The photoreceptor membranes of DEF showed disarrangement of the lamellar structures of one-third of the apical portion (B). Inner segments showed no mitochondrial swelling or other morphological changes in either group. Bar: 2 μ m.

showed disarrangement of the lamellar structures in one-third of the apical portion (Figure 2B). The photoreceptor membrane of the SUP rats was normal (Figure 2A). No mitochondrial change was observed in the inner segments in either group. The nuclei of the photoreceptor cells were normal.

After a 3-hour exposure, the mitochondria in RPE and photoreceptor membranes showed more extensive changes in the DEF rats than the SUP rats. After a 6-hour exposure, morphological changes of the RPE developed in both groups, including flattening of the cells and shortening of basal infoldings. Marked tubovesiculation of the photoreceptor membrane was observed in both groups.

After a 12-hour exposure, there was no significant morphological difference in RPE and photoreceptor cell between the groups. In both, some photoreceptor cell nuclei contained uniformly dense chromatin, apparently pyknotic.

After a 24-hour exposure, nearly 60% of photoreceptor cell nuclei in the SUP rats (Figure 4A), but almost 90% in the DEF rats (Figure 4B) were pyknotic. Very electron-dense particles, seemingly apoptotic bodies, were scattered around the pyknotic cells in both groups and were phagocytosed by neighboring photoreceptor cells.

After a 72-hour exposure, there were some morphologically normal RPE cells in both groups but

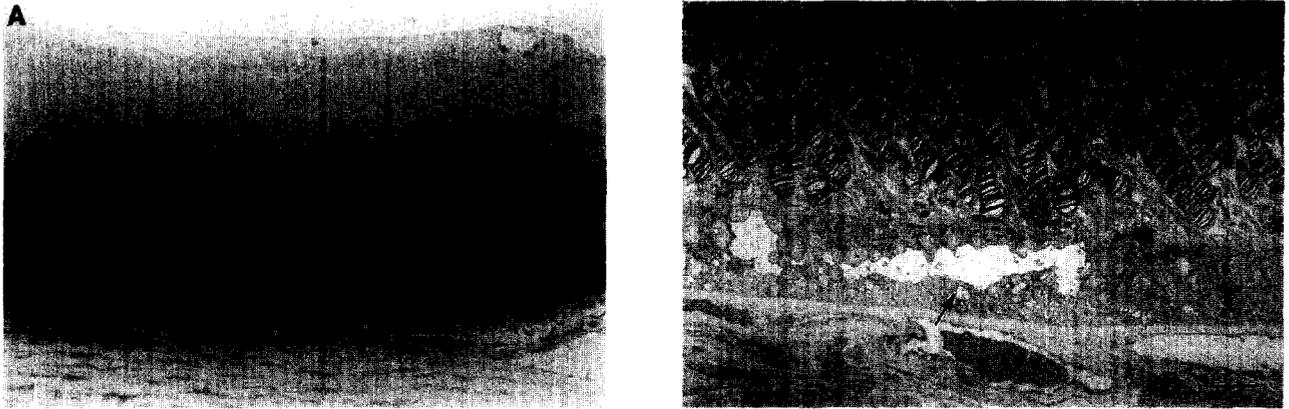


Figure 3. (A) Light micrograph (toluidin blue stain $\times 400$) (B) Electron micrograph. After 1-hour light exposure, RPE of DEF group showed vacuolation of the cytoplasm (arrows). Bar: 2 μm .

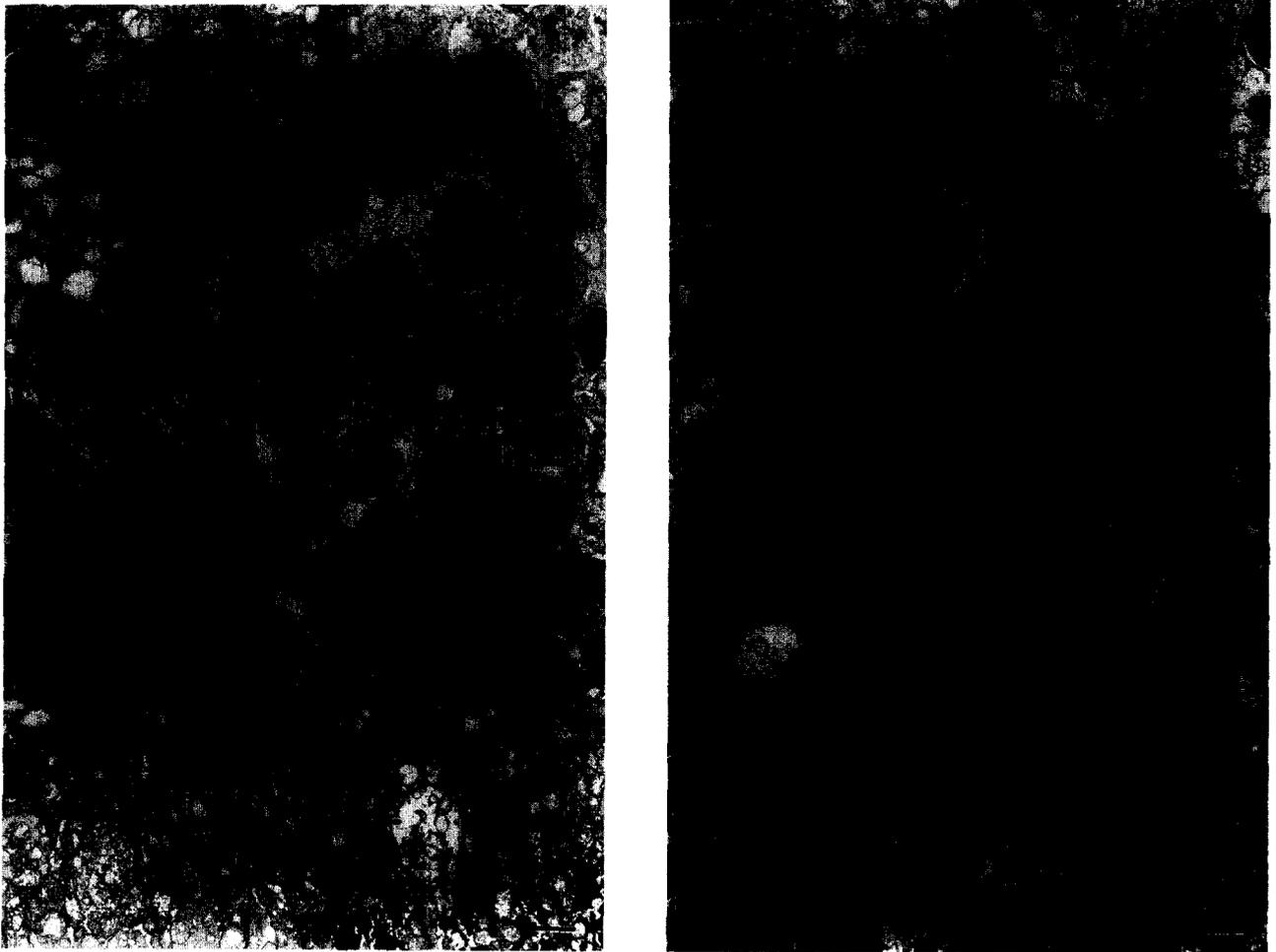


Figure 4. After 24-hours exposure. Sixty percent of photoreceptor cell nuclei in SUP were pyknotic (A), 90% in DEF (B). Electron-dense particles are scattered around the pyknotic cells in both groups. Bar: 2 μm .

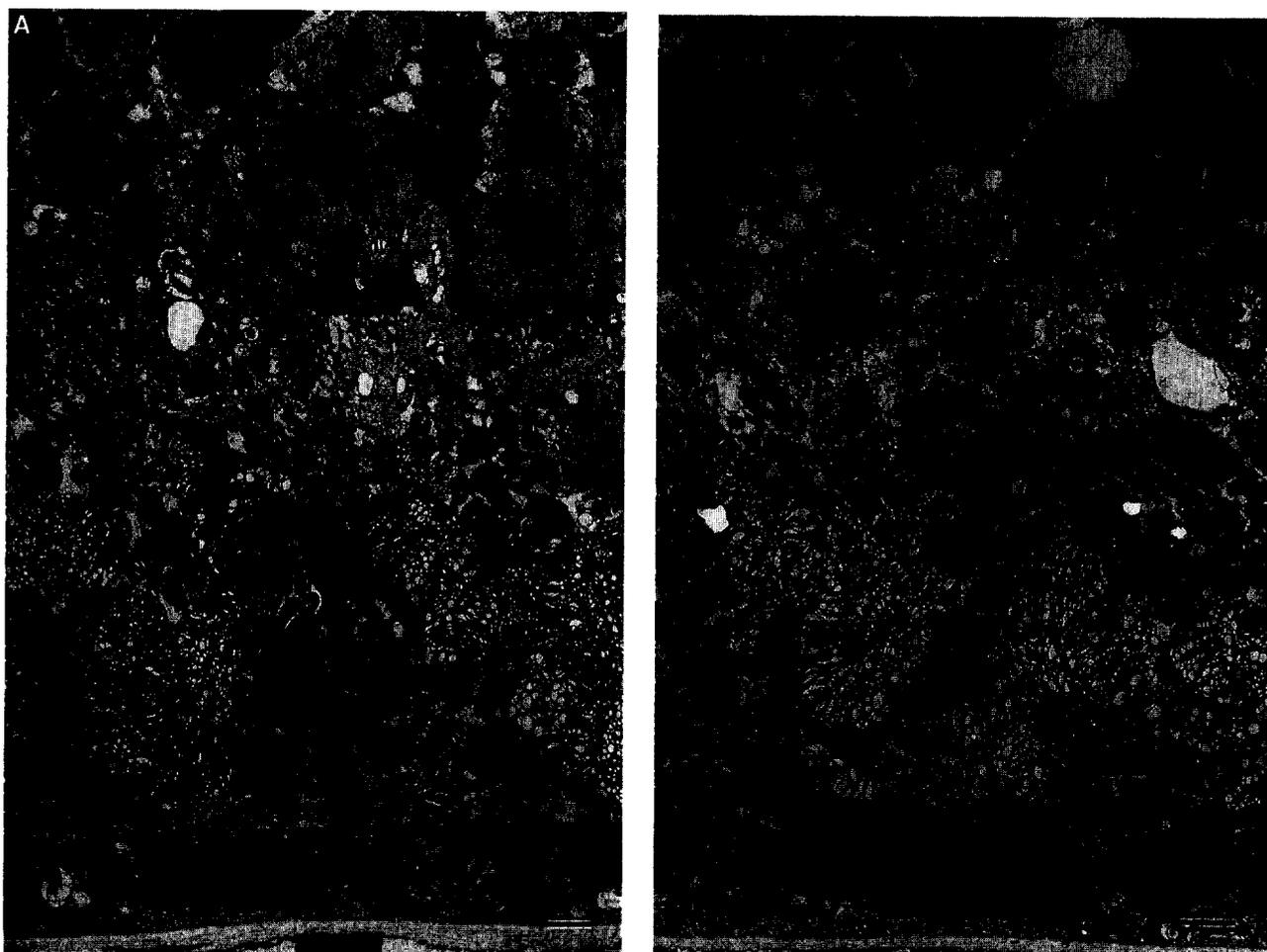


Figure 5. After a 72-hour exposure, RPE had large electron-dense phagosomes without lamellar structure. Some outer segments showed condensation of membrane, others showed vesicular changes, in both groups (**A:** SUP, **B:** DEF). Bar: 2 μm .

the RPE had large electron-dense phagosomes and without lamellar structure. Some outer segments showed condensation of the membrane and others showed vesicular changes in both groups (Figures 5A and 5B). Findings differed by area of specimen. The inner retinal layer showed no significant changes in either group at any exposure.

Image-Analysis of Phagosomes in RPE

Results are shown in Figure 6. There were more large phagosomes in SUP than DEF rats after a 1-hour exposure and more in DEF than SUP rats after 3-, 6-, and 12-hour exposures. After 24 and 72 hours, there is no significant difference in the groups. There were more small phagosomes in the SUP than the DEF rats after a 1-hour exposure and in the DEF, rather than the SUP rats, after 6, 12, and 72 hours of exposure.

Discussion

It has been proposed that α -tocopherol functions as a protective factor against peroxidation of fatty acids and restrains breakdown of cell membranes and release of lysosomal enzymes.^{13,19} Its effectiveness in prevention of retinal light damage was previously reported histologically in pigmented rabbits exposed to xenon light without long waves.^{10,11} However, the independent action of α -tocopherol is described as less effective.^{12,13} Katz and Eldred reported that vitamin E deficiency did not enhance the effect of bright cyclic light (750 lux) in reducing photoreceptor cell densities in the rat retina.⁹ Stone et al reported that dietary vitamin E and selenium deficiencies failed to enhance the autofluorescent pigment in RPE and electroretinogram amplitudes in rats exposed to fluorescent light.¹⁴ In our previous study, we exposed rats to the light for 12 hours, kept

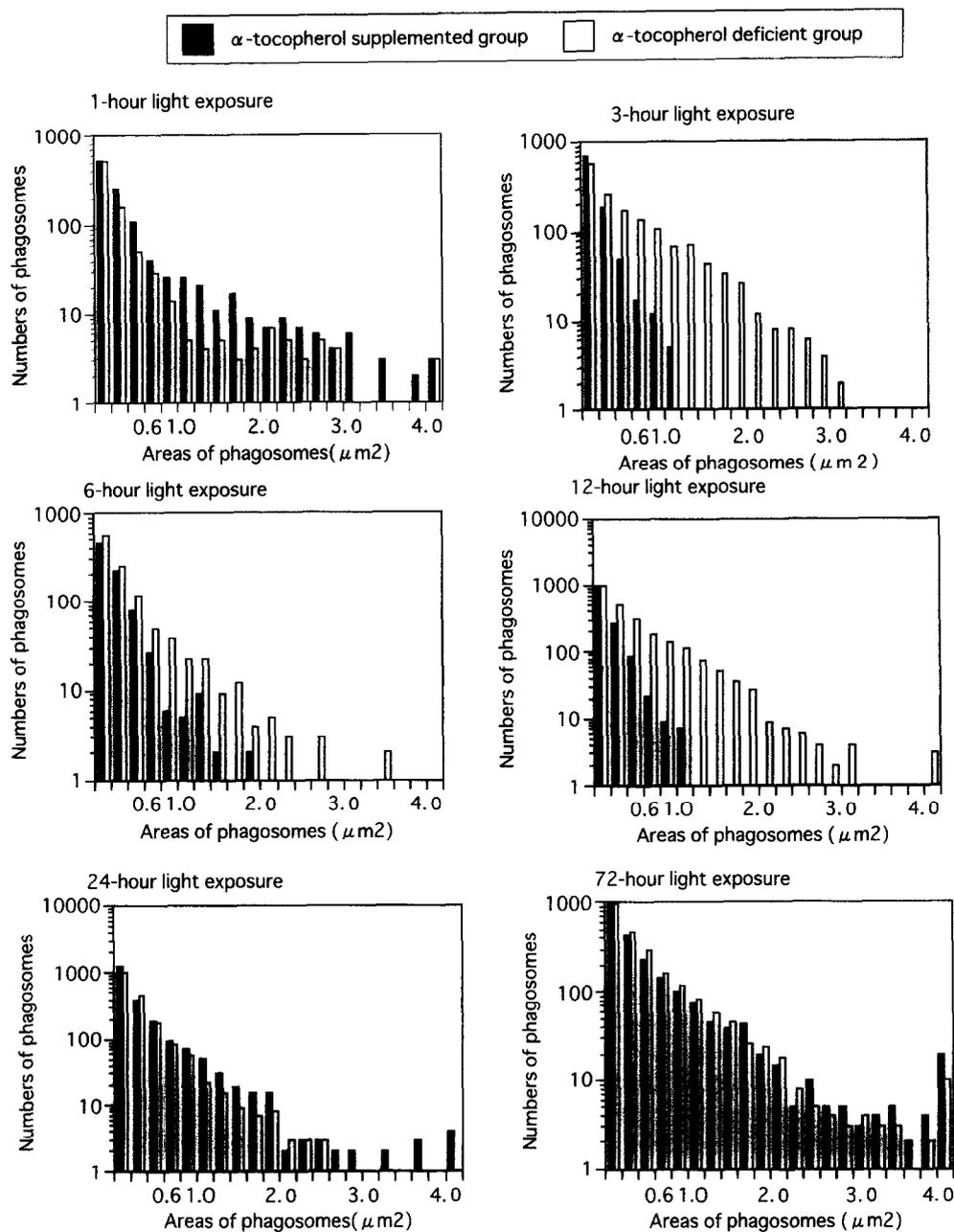


Figure 6. Image analysis of phagosomes in RPE. There were more large phagosomes ($>0.6 \mu\text{m}^2$) in the SUP than DEF rats after a 1-hour exposure and more in the DEF than the SUP group after 3-, 6-, and 12-hour exposures. After 24 and 72 hours, there was no significant difference in the groups.

them in the dark for 2 weeks and found no significant difference between the DEF and SUP groups, using electroretinograms and measurement of outer nuclear layer thickness/whole retinal layer thickness with light microscopy.¹⁵

In the present study, we examined the ultrastructural changes induced by constant light of relatively

short duration (1–72 hours). No retinal changes have been shown, previously, in rats fed a vitamin E deficient diet for 3 months.¹⁹ Our study also found no retinal changes in unexposed rats fed an α -tocopherol deficient diet for 8 weeks or a supplemented diet. After 1- and 3-hour exposures, the changes in photoreceptor membranes and RPE cells were more

marked in the DEF than in the SUP group. However, these changes were similar in both groups after a 12-hour exposure. After a 24-hour exposure, photoreceptor cells with pyknotic nuclei were 60% in the SUP rats and 90% in the DEF rats. Cell death was identified by findings of apoptosis.

Morphological changes in the RPE were observed after a 1-hour exposure but progressed very little with increased exposure. Normal RPE cells were present after 72 hours of exposure. Damage of photoreceptor cells increased as exposure was prolonged. These findings corresponded with the photochemical damage reported by Noell et al.²⁰ These findings and the spectrum of the light source indicate that our results differed from those produced by short-wavelength light.²¹

LaVail counted large phagosomes as a measure of disk shedding and thought that small inclusions below criterion size probably were degraded stages of large phagosomes.¹⁸ In this study, image analysis of phagosomes showed that the logarithm of the number of phagosomes decreased almost linearly as their area increased. There was no distinct borderline between small and large phagosomes. The volume of phagosomes in RPE is probably determined by intake and digestion of shedding disks by the pigment epithelium. End products of undigested disks are thought to remain in the RPE as lipofuscin granules.^{7,12,14} We did not study the autofluorescence peculiar to lipofuscin, but, the electron-dense large phagosomes without lamellar structure, seen after 24 or 72 hours of exposure, may be identified as lipofuscin granules. Because lipofuscin is not strictly differentiated from phagosomes, we regarded all inclusions stained by toluidine blue in the somas and microvilli of RPE as "phagosomes." After a 1-hour light exposure, there were more large phagosomes in the SUP than the DEF, probably suggesting that intake by RPE decreased in the DEF more than the SUP. However, burst of disks shedding with the circadian rhythm could influence the results. After 3-, 6-, and 12-hour exposures, large phagosomes were more numerous in the DEF than the SUP rats, but there was no significant difference in changes in the outer nuclear layers and outer segments in the groups. These results seem to reflect the reduced digestibility of RPE in the DEF rat. After 24- and 72-hour exposures, large phagosomes increased in both groups, and the morphological changes in the outer nuclear layer, outer segments, and RPE were greater. RPE cells were atrophic in some places and hypertrophic in others, so that it was difficult to evaluate the analysis of phagosomes after 24 and 72 hours of exposure.

In summary, (a) after 1- and 3-hour exposures, morphological changes in the RPE and photoreceptor membranes were more extensive in the DEF than the SUP groups; (b) after a 24-hour exposure, there were more pyknotic nuclei in photoreceptor cells of the DEF than SUP groups; and (c) after 3-, 6-, and 12-hour exposures, there were more phagosomes in the RPE of the DEF than the SUP rats. This suggests that α -tocopherol protects the retina from light injury for up to 24 hours of exposure. It was a surprise to find no significant difference in the DEF and SUP groups after 72-hours of exposure despite 80 times the amount of α -tocopherol in the neural retina of the SUP group than the DEF rats. These results may be associated with a decrease in other free radical scavengers. Phagosome image-analysis of the RPE was useful for examining retinal light damage; we will use this technique to study the protective effect of other agents.

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