

Melanin Protects Choroidal Blood Vessels against Light Toxicity

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Z. Naturforsch. **61c**, 427–433; received January 12/February 24, 2006

Low ocular pigmentation and high long-term exposure to bright light are believed to increase the risk of developing age-related macular degeneration (ARMD). To investigate the role of pigmentation during bright light exposure, cell damage in retinæ and choroids of pigmented and non-pigmented rats were compared. Pigmented Long Evans (LE) rats and non-pigmented (albino) Wistar rats were exposed to high intensity visible light from a cold light source with 140,000 lux for 30 min. Control animals of both strains were not irradiated. The animals had their pupils dilated to prevent light absorbance by iris pigmentation. 22 h after irradiation, the rats were sacrificed and their eyes enucleated. Posterior segments, containing retina and choroid, were prepared for light and electron microscopy. Twenty different sections of specified and equal areas were examined in every eye. In albino rats severe retinal damage was observed after light exposure, rod outer segments (ROS) were shortened and the thickness of the outer nuclear layer (ONL) was significantly diminished. Choriocapillaris blood vessels were obstructed. In wide areas the retinal pigment epithelium (RPE) was absent in albino rats after irradiation. In contrast, LE rats presented much less cell damage in the RPE and retina after bright light exposure, although intra-individual differences were observed. The thickness of the ONL was almost unchanged compared to controls. ROS were shortened in LE rats, but the effect was considerably less than that seen in the albinos. Only minimal changes were found in choroidal blood vessels of pigmented rats. The RPE showed certain toxic damage, but cells were not destroyed as in the non-pigmented animals. The number of melanin granules in the RPE of LE rats was reduced after irradiation. Ocular melanin protects the retina and choroid of pigmented eyes against light-induced cell toxicity. Physical protection of iris melanin, as possible in eyes with non-dilated pupils, does not seem to play a major role in our setup. Biochemical mechanisms, like reducing oxidative intracellular stress, are more likely to be responsible for melanin-related light protection in eyes with dilated lens aperture.

Key words: Melanin, RPE, Light Toxicity

Introduction

Exsudative age-related macular degeneration (ARMD), the major cause of blindness of the elderly in the industrial world, occurs more than twice as often in Caucasians than in black Africans (Gregor and Joffe, 1978). Significant associations between iris color, fundus pigmentation and ARMD suggest that melanin plays a crucial role in the development of this disease (Weiter *et al.*, 1985). Increased exposure to bright light is known to impair morphology and function of the retina and the retinal pigment epithelium (RPE). As melanin in the RPE cannot act as a physical sun screen like the iris melanin does, it has been suggested that melanin in the RPE acts photo-protective

by scavenging free radicals (Young, 1988). But still the role of RPE melanin is not entirely clarified and pro-oxidative features of the melanin molecule have also been described (Sarna, 1992). To determine the role of melanin pigmentation, we investigated phototoxic damages in retinal and choroidal tissue of pigmented (Long Evans) and albino (Wistar) rats.

Materials and Methods

Illumination

Wistar (albino) and Long Evans (LE, pigmented) rats were exposed to constant bright visible light (380–625 nm) provided by a cold light source (KL 1500 LCD, Schott, Glas Fiber Optics

Division, Germany) with light guides for 30 min. Light intensity was 140,000 lux, measured with a photometer (Colormaster 3F, Gossen, Erlangen, Germany) at the corneal surface. Before irradiation, the rats' pupils were dilated with phenylephrin-tropicamide eye drops to avoid light absorbance by iris pigmentation. The animals were fixed in the same position over the whole assay time, providing a constant distance (5 mm) and angle between cornea and light source. This way, light intensity was evenly spread in every single eye and all regions of the eyecup were equally irradiated in all animals. Temperature was measured at regular intervals and the corneas were repeatedly moistened with saline solution to prevent corneal burns. Following light exposure, the animals were kept in room conditions and sacrificed 22 h after illumination.

All procedures involving animals were performed according to policies for the use of animals in research.

Preparation and fixation of the eyes

The eyes were enucleated and the anterior segment was removed at the equator. From each posterior segment equal topographic areas were prepared for analysis, in order to obtain comparable results in case of local differences in light sensitivity. Twenty different sections were chosen per eye. Pieces of 2 mm in diameter were cut with their center 2 mm above the upper margin of the optic disc. The tissue pieces thus obtained were fixed in 0.1 M cacodylate buffer (pH 7.4, VWR international, Darmstadt, Germany) containing 3% glutaraldehyde (Plano, Wetzlar, Germany) overnight at 4 °C and then post-fixed at room temperature in 0.2 M cacodylate buffer containing 1.5% osmiumtetroxide for 2 h. Samples were stained in uranyl acetate (Plano) containing 70% ethanol overnight. After dehydration in graded series of ethanol, samples were embedded in Spurr's resin and incubated at 70 °C for 3 d.

Light microscopy

Semithin sections (500 nm) were cut using a microtome (Ultracut-E-Reichert Jung, Germany) and stained with methylene blue. In every section the maximal thickness and length of nuclei, rod outer segments (ROS) and RPE layer were measured.

Electron microscopy

Ultrathin sections (70 nm) were cut and then stained with uranyl acetate and lead citrate to quantify the amount of pigment granules using a transmission electron microscope (Zeiss 902A, Zeiss, Oberkochen, Germany).

Morphologic and morphometric analysis

The morphologic and morphometric analysis (12 measurements for each pigmentation, $n = 12$) was performed by a light microscope (Axioplan, Zeiss) connected to a personal computer equipped with a video camera module (Mod. ORCA-ER C4742-95, Hamamatsu Photonics, Hamamatsu City, Japan). In every semithin section three areas at intervals of 500 μm were used and analyzed for quantification. If in one of the areas thus determined no quantification was possible due to an absence of cells as a result of the light damage, the adjacent area was measured. A commercial software package (Improvision, Openlab, Coventry, England) was used for quantification. Statistical analysis was based on Student's t-test for two populations.

Results

Electron microscopy

Electron microscopy revealed significant differences in the degree of damage in the choroid and retina of albino and pigmented rat retinas after irradiation. Whereas pigmented LE rats only showed moderate damage, albino rats were characterized by wide areas of RPE and photoreceptor destruction, in addition to obstructed choriocapillaris vessels.

Long Evans rats after irradiation

LE rats often presented wide areas of undamaged or only minimally damaged tissue after irradiation (Fig. 1A), with well preserved photoreceptor outer segments, RPE cells expressing intact microvilli, containing their typical spindle-shaped melanin granules and phagosomes at the cell apex, as well as an intact basal labyrinth. The choriocapillaris vasculature was adequately perfused (Fig. 1B).

In some areas, however, a destruction of RPE cells became evident. An example of the worst observed damage in LE rats is shown in Fig. 1C. These damaged RPE cells typically contained ag-

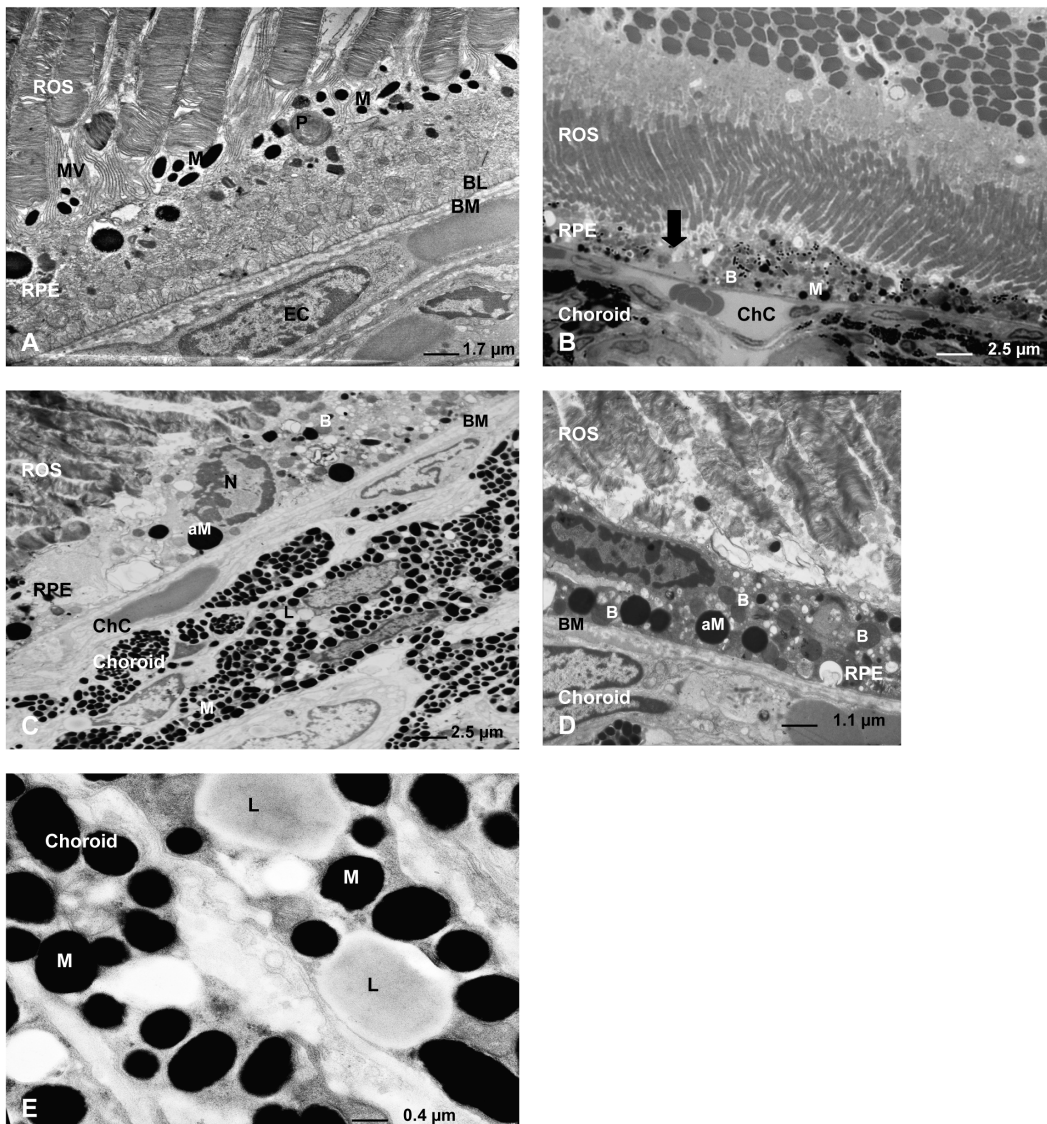


Fig. 1. LE rats after irradiation: electron micrographs of retina-choroid complexes. (A) LE rats show wide areas of very healthy looking tissue after irradiation. Photoreceptor outer segments (ROS) are well preserved. The RPE looks healthy with its microvilli (MV), its typical spindle-shaped melanin granules (M) and phagosomes (P) at the cell apex and the basal labyrinth (BL). No evident signs of destruction are present. BM, Bruch's membrane; EC, endothelial cell of choriocapillaris. (B) This electron micrograph shows sufficiently healthy looking ROS and a well perfused choriocapillaris (ChC) vasculature in LE rats after irradiation. But there is damage in the RPE cell layer, showing loss and destruction of RPE cells (arrow). (C) In some cases LE rats showed severe damage after irradiation. The photoreceptor outer segments (ROS) were altered and nuclei (N) of RPE cells appear destroyed. A striking characteristic of irradiated RPE is the loss of typical RPE sphere-shaped melanin granules at the cell apex. Instead one can observe large round granules of agglutinated melanin (aM) at the bottom of the cell. There are large electron dense inclusion bodies (B) in the RPE. The choroid is characterized by large lipid-like electron-opaque drops (L) within the melanocytes, which have not been described before. In contrast to the albino rats occluded choriocapillaris (ChC) vessels in LE rats after irradiation are a rarity. BM, Bruch's membrane. (D) Photoreceptor outer segments (ROS) are still present but look stressed. Again the loss of RPE-typical melanin is striking, instead there are electron-dense bodies (B) and huge melanin agglutinates (aM) at the basal side of the cell. BM, Bruch's membrane. (E) High magnification of the choroid of a LE rat shows large electron-opaque, lipid-like droplets (L) within choroidal melanocytes, so far not described before.

glutinations of melanin granules at the basal side of the cell (Figs. 1B, D). The number of typical spindle-shaped melanin granules in the RPE was markedly reduced (Figs. 1B–D). Electron dense inclusion bodies in the RPE (Figs. 1B–D) were observed after irradiation. Only occasionally, obstructed choriocapillaris vessels were seen in LE rats (Fig. 1C). Destruction of photoreceptor outer segments was also found to a certain extent, but not as severe as in albino rats. The most prominent finding in the choroid of LE rats after irradiation was the presence of large electron-opaque, lipid-like drops inside of melanocytes (Fig. 1E), which has never been described before.

Wistar (albino) rats after irradiation

Wistar rats showed extended damage after irradiation (Fig. 2). Photoreceptor outer segments were usually completely destroyed, instead there were inflammatory cells invading the retina and choroid. Extravascular red blood cells indicated severe damage of the retinal and choroidal vasculature (Fig. 2A). In wide areas the RPE was severely destroyed (Fig. 2A) or completely absent (Figs. 2B, C), leaving behind a denuded Bruch's membrane. The remaining RPE cells showed large electron-opaque inclusion bodies (Figs. 2A, B), similar to those observed in LE rats after irradiation. In the albino rats, some of these bodies were located extracellularly in the retina. In wide areas, choriocapillaris blood vessels were obstructed by clots of red blood cells (Fig. 2C).

LE rats: thickness of the outer nuclear layer (ONL) and rod outer segments (ROS)

Thickness of the outer nuclear layer in untreated LE rats was $(34.20 \pm 3.32) \mu\text{m}$. The length of rod outer segments was $(34.65 \pm 8.35) \mu\text{m}$ (mean values). After irradiation, the ONL was $(31.61 \pm 7.28) \mu\text{m}$ thick and ROS were $(25.83 \pm 5.17) \mu\text{m}$ in length (mean values, Table I). Whereas the thickness of the ONL was not significantly reduced after irradiation in LE rats ($p = 0.27$), ROS were significantly shortened ($p = 0.005$).

Wistar (albino) rats: thickness of ONL and ROS

Thickness of the outer nuclear layer in untreated Wistar rats was $(35.80 \pm 6.62) \mu\text{m}$. The length of rod outer segments was $(38.93 \pm 3.62) \mu\text{m}$ (mean values, Table I). After irradiation, the ONL was only $(18.02 \pm 18.87) \mu\text{m}$ thick and

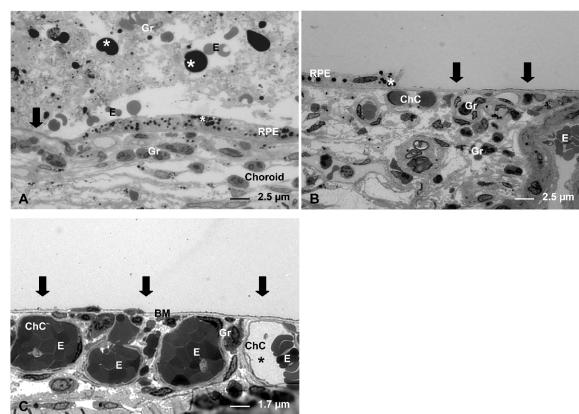


Fig. 2. Albino (Wistar) rats after irradiation: electron micrographs of the retina-choroid complexes. (A) Wistar rats show extended damage after irradiation: photoreceptor outer segments are completely destroyed, instead there are inflammatory cells (Gr, granulocytes) invading the retina and choroid. Scattered red blood cells (E) indicate severe damage of the retinal and choroidal vasculature. In some areas the RPE is missing, leaving behind a denuded Bruch's membrane (arrow). The huge electron-dense granules (asterisks), seen in the retina and as smaller particles in the RPE, are most likely lysosomal residues from an overload of destroyed and incompletely degraded cellular material after irradiation. There are no microvilli on the RPE cells. (B) A common finding of irradiated Wistar rats was the loss of RPE cells in wide areas (arrows). The remaining RPE cells accumulate large electron dense bodies (asterisk). Choriocapillaris (ChC) blood vessels are often not perfused due to thrombosis. Granulocytes (Gr) have invaded the choroid. E, erythrocytes in a large choroidal blood vessel. (C) The RPE cell layer is completely destroyed in this section of an albino rat after irradiation. Choriocapillaris (ChC) vasculature is severely obstructed by clots of red blood cells (E), only little perfusion remains open (asterisk). Granulocytes (Gr) have invaded the choroid. Loss of the photoreceptor layer is more likely artefact-related.

ROS were shortened to $(10.50 \pm 12.44) \mu\text{m}$. Statistical analysis showed that the thickness of the ONL was significantly reduced ($p = 0.005$) and ROS were significantly shorter after irradiation ($p < 0.0005$).

Thickness of ONL and length of ROS in LE rats compared to Wistar rats

Comparison of the outer nuclear layer and the length of ROS between LE and Wistar rats after irradiation showed that pigmented rats have significantly less light induced retinal damage than the albinos [$p(\text{ONL}) < 0.05$; $p(\text{ROS}) < 0.005$; Table I].

Table I. Comparison of light-induced effects between pigmented and non-pigmented rats. In LE (pigmented) rats thickness of the ONL had not significantly changed after irradiation, whereas ROS were significantly shortened. In Wistar (albino) rats ($n = 12$) the thickness of the ONL and of ROS was significantly reduced after irradiation (ONL: $p = 0.005$; ROS: $p < 0.0005$). The thickness of ONL and ROS in Wistar rats was reduced compared to LE rats [ONL (LE/Wistar): $p < 0.05$; ROS (LE/Wistar): $p < 0.005$]. The obstruction rate of choriocapillaris blood vessels in albinos was significantly higher than in pigmented animals. The number of melanin granules in the RPE was reduced from 0.18 per μm^2 RPE cell without irradiation to 0.09 after irradiation. Untreated RPE contained high numbers of typical spindle-shaped melanin granules and less spherical melanosomes. After irradiation, this proportion of spindle-shaped and spherical melanosomes switched significantly to a higher proportion of spherical granules, due to a selective loss of spindle-shaped melanin granules.

	LE control	LE irradiated	p	Albino control	Albino irradiated	p
Thickness of ONL [μm]	34.20	31.61	0.27	35.80	18.02	0.005
Thickness of ROS [μm]	34.65	25.83	0.005	38.93	10.50	0.0005
Obstructed choriocapillaris vessels (No. per 0.5 mm section)	–	0.7	–	–	3.4	–
Number of melanin granules per m^2	Spindle: 0.09 Spherical: 0.09 Total: 0.18	Spindle: 0.03 Spherical: 0.07 Total: 0.09	< 0.05	–	–	–

Surviving RPE on Bruch's membrane (BM)

Measuring the dimensions of surviving RPE on Bruch's membrane (in % RPE coverage of total BM) in albino and pigmented rats after irradiation, it is shown that the extent of RPE destruction is about twice as high in albino rats than in pigmented rats. If the mean length of RPE cover in irradiated LE rats is set at 100%, the surviving RPE length on BM in albino rats is only 52.63% (± 38.71), corresponding to a significant ($p = 0.0003$) reduction of RPE length on BM.

Obstruction of choriocapillaris blood vessels in albino and pigmented rats

Obstruction of choriocapillaris blood vessels was predominantly seen in albino rats (Fig. 2C, Table I), barely in pigmented rats. The number of obstructed vessels per 0.5 mm sectioned choroid in LE rats was 0.7 (± 1.0), in Wistar rats 3.4 (± 1.9) (LE/Wistar $p < 0.0004$).

Quantification of distinct melanosomes forms in the RPE of irradiated and untreated LE rats

Irradiation of pigmented rats led to a loss of RPE cell melanin (Figs. 1C, D, Table I). The number of melanin granules in the RPE was reduced from 0.18 (± 0.10) per μm^2 RPE cell without irradiation to 0.09 (± 0.07) after irradiation ($p = 0.027$) (Table I).

Analyzing the proportion of different shapes of these melanin granules with and without irradiation, the following observation was made: untreated RPE usually contained high numbers of typical spindle-shaped melanin granules (Fig. 1C, Table I) and additionally, spherical melanosomes. After irradiation, this proportion of spindle-shaped and spherical melanosomes switched significantly to a higher proportion of spherical granules, due to a selective loss of spindle-shaped melanin granules.

Discussion

The question, whether ocular and particularly RPE melanin is more protective or more destructive for the retinal tissue is still unsettled. RPE or choroidal melanin does not act as a physical protection shield for photoreceptors as it is situated beyond them in the eye. So if this melanin was supposed to be photo-protective, its main mode of protection would most likely be related to its antioxidant capacity (Sarna, 1992).

To rule out physical light protection of iris melanin in pigmented rats, their pupils were dilated before irradiation. Our electron microscopic and quantitative analyses demonstrate that pigmented rats are significantly better protected against light damage than albino rats when irradiated with the same dose and duration of white light from a cold

light source. The high light intensity was chosen according to a protocol of Kayatz *et al.* (1999), demonstrating only moderate damage in pigmented animals with these parameters. As cold light does not, or only minimally (1 °C), increase retinal temperature (Noell *et al.*, 1966), the damage observed was only related to light, not caused by thermal effects. We used white light for irradiation, as this has the highest relevance in daily life, *e.g.* for patients with ARMD having been exposed to increased light during their lifetime, or for patients during ophthalmic surgery. Several studies indicate, that melanin-related effects depend on the wavelength of the light, but there is disagreement about the wavelength which is protective or deleterious for (Collier *et al.*, 1989; Gorgels and Van, 1998; Rapp and Smith, 1992). The mediator of photodamage may be rhodopsin and/or other chromophores (Noell *et al.*, 1966). The reason why our visible daylight does not have more deleterious effects in terms of phototoxicity and ARMD incidence was presumed to be due to the cornea and lens filtering most of the light below 400 nm (Boettner and Wolter, 1962).

Pigmented rats in our study were characterized by significantly more surviving RPE and photoreceptor cells and a far better perfused choriocapillaris than albino rats. In regions with destroyed photoreceptors, the RPE was also severely damaged and *vice versa*, although variations between different sections were observed and responsible for the high standard deviations in the quantitative analyses. A combination of reasons is held responsible for the significant shortening of outer segments, which was particularly seen in albino rats: the bright light induces enhanced disc shedding and more breakage of outer segments (Kuwabara, 1979) which leads together with a reduced capacity for disc membrane renewal by irradiation-damaged RPE and photoreceptor cells to the observed thinning of the outer segment layer. The particularly in albinos observed thinning of the outer nuclear layer reflects photoreceptor cell death. A bare Bruch's membrane without any covering RPE cells was seen twice as often in albinos than in pigmented rats. On increasing doses of light the RPE and the outer segments are damaged prior to the other retinal cells (Kuwabara, 1979). As the RPE is in part responsible for the maintenance of the photoreceptors, the light-induced damage on the retina is not only directly induced by the light

exposure but also by a dysfunction of the underlying RPE.

The oxidative stress caused by the irradiation in retinal and choroidal cells is enhanced by the particularly high perfusion rate of the choroid (Parver *et al.*, 1980). Oxidative stress causes lipid peroxidation in cell membranes leading to dysfunction of organelles and the cells themselves. Our results support the hypothesis that melanin of pigmented strains acts photo-protective (Noell *et al.*, 1966; Sanyal and Zeilmaker, 1988; Memoli *et al.*, 1997). Melanin competes with superoxide dismutase for the scavenging of superoxide radicals (Korytowski *et al.*, 1985), the melanin molecule is being autooxidized and hydrogen peroxide is produced. The zinc supply bound to the melanin can be used as a co-factor for anti-oxidative enzymes, such as carboanhydrase or superoxide dismutase (Rimbach *et al.*, 1996). The melanin-related metabolites 5,6-dihydroxyindole (DHI), 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and 5-*S*-cysteinyl-dopa (CD) were found to be more potent inhibitors of lipid peroxidation than ascorbic acid or glutathione (Memoli *et al.*, 1997). Being diffusible substances, these compounds are likely to carry out their antioxidative role not only in the RPE but also in surrounding tissue like the retina. The large electron-dense inclusion bodies seen in the destroyed retina of albinos as well as the smaller inclusion bodies in the RPE (Fig. 2) are supposed to be residues from insufficiently degraded disc membranes of oxidatively damaged photoreceptor outer segments. Peroxidized lipids in disc membranes inhibit their lysosomal digestion, leading to the formation of lipofuscin (Kuwabara, 1979). Membranous destabilization might also be the explanation for the so far not described large electron-opaque lipid-like drops observed in choroidal melanocytes of pigmented rats (Fig. 1E), although the exact origin of these drops is not known. The large conglomerates of melanin seen in the RPE of pigmented rats and the shift of shape towards more spherical than spindle-shaped melanin granules (Table I) may be either a result of oxidative changes in the organelle membrane or be related to chemical changes and fragmentation of the melanin molecule, as described by Kuwabara (1979).

The diffuse invasion of inflammatory cells in albino rats and the extravasation of red blood cells can be explained by an oxidative stress related damage of the vessel wall. Arterial and venous

thrombosis, as seen predominantly in the choriocapillaris of albinos can be induced by oxidative stress (Undas *et al.*, 2005) and would further affect the nutrition of the outer layers of the retina.

However, the antioxidative properties of melanin were doubted by some authors (Glickman and Lam, 1992; Putting *et al.* 1994), leading Sarna (1992) to the hypothesis that melanin predominantly quenches radicals generated inside the melanosomes itself. As melanin binds cell toxic metal ions, *e.g.* copper and iron, with high affinity, the main function of melanin may be to deactivate these peroxidizing agents. As exposure of synthetic melanin to intense fluxes of highly oxidizing

species was found to reduce the molecule's antioxidative capabilities (Burke *et al.*, 1991), it was presumed that photo-oxidized RPE melanin could also lose its antioxidant efficiency and even become pro-oxidant, due to a release of redox-active metal ions into the cytoplasm (Sarna, 1992).

However, our results strongly suggest that at the end RPE melanin acts photo-protectively on irradiation with very high light intensities. This would be in accordance with the fact that ARMD is significantly less prevalent in individuals with dark pigmentation. Retinal as well as choroidal tissue is far less damaged by irradiation in pigmented rats than in albino rats.

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