

Retinal pigment epithelium, age-related macular degeneration and neurotrophic keratouveitis

ENRICA BIANCHI¹, FABIO SCARINCI², GUIDO RIPANDELLI², JANOS FEHER¹, ELENA PACELLA¹,
GIUSEPPE MAGLIULO¹, CORRADO BALACCO GABRIELI¹, ROCCO PLATEROTI¹,
PASQUALE PLATEROTI¹, FIORENZO MIGNINI³ and MARCO ARTICO¹

¹Department of Sensory Organs, University of Rome 'La Sapienza', Rome; ²Bietti Eye Foundation IRCCS, Rome;

³School of Drug and Health Products Sciences, University of Camerino, Camerino, Italy

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Abstract. Age-related macular degeneration (AMD) is the leading cause of impaired vision and blindness in the aging population. The aims of our studies were to identify qualitative and quantitative alterations in mitochondria in human retinal pigment epithelium (RPE) from AMD patients and controls and to test the protective effects of pigment epithelium-derived factor (PEDF), a known neurotrophic and antiangiogenic substance, against neurotrophic keratouveitis. Histopathological alterations were studied by means of morphometry, light and electron microscopy. Unexpectedly, morphometric data showed that the RPE alterations noted in AMD may also develop in normal aging, 10-15 years later than appearing in AMD patients. Reduced tear secretion, corneal ulceration and leukocytic infiltration were found in capsaicin (CAP)-treated rats, but this effect was significantly attenuated by PEDF. These findings suggest that PEDF accelerated the recovery of tear secretion and also prevented neurotrophic keratouveitis and vitreoretinal inflammation. PEDF may have a clinical application in inflammatory and neovascular diseases of the eye.

Introduction

Age-related macular degeneration (AMD) is a progressive neurodegenerative disease of the central retinal area (macula lutea) and it represents the most common cause of legal blindness in industrialized countries (1,2). Epidemiologic studies from several countries also showed a dramatic increase in the prevalence and severity of AMD with age. Despite intensive basic and clinical research, its pathogenesis remains unclear,

likely due to its multifactorial character (3-6). In addition to a strong age-dependence of the disease, a complex interaction of metabolic, functional, genetic and environmental factors appears to create a platform for chronically developing changes in the ocular structures of the macular region [chorio-capillaries, Bruch's membrane, retinal pigment epithelium (RPE), photoreceptors] which may contribute in varying degrees to the onset of AMD.

Traditionally, two subgroups of AMD can be distinguished: atrophic (dry form) and exudative (wet form). The dry form, synonymously known as age-related maculopathy, is characterized by the presence of small yellowish deposits (drusen) under the RPE, accompanied with either loss or focal accumulation of melanin pigment. This form of AMD is typically characterized by a progressing course leading to degeneration of RPE and photoreceptors. The exudative form is linked to choroidal neovascularization directed to the subretinal macular region, with subsequent bleeding and/or fluid leakage, which may result in a sudden loss of central vision; it is the most rapidly progressing form of AMD. Both atrophic and exudative forms are associated with severe impairment of visual functions (7). The pathophysiology of AMD is complex and, in addition to genetic predispositions, at least 4 processes contribute to the disease: lipofuscinogenesis, drusogenesis, local inflammation and neovascularization (in the case of the wet form) (3-13). The current pathophysiologic concept regarding AMD assigns a primary role to the age-related, cumulative oxidative damage to the RPE due to an imbalance between generation and elimination of reactive oxygen species (ROS) (14-16). Specifically, lipofuscin, a heterogeneous material composed of a mixture of lipids (lipid peroxides, proteins and different fluorescent compounds derived mainly from vitamin A), has been hypothesized to be the primary source of ROS responsible for both cellular and extracellular matrix alterations in AMD (17-19).

Accumulation of lipofuscin, other lipid peroxides and potentially toxic substances may dramatically influence the RPE physiology. *In vitro* it greatly reduces the phagocytic capacity, lysosomal enzyme activities and antioxidant potential of human RPE (20,21). *In vivo* studies support findings regarding the *in vitro* effects of lipofuscin on RPE metabolism (22). N-retinylidene-N-retinylethanolamine (A2E), the major

Correspondence to: Dr Enrica Bianchi, Department of Sensory Organs, University of Rome 'La Sapienza', Viale del Policlinico 155, 00161 Rome, Italy
E-mail: enrica.bianchi@uniroma1.it

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autofluorescent component of lipofuscin, specifically targets cytochrome *c* oxidase (23,24), causes caspase activation and RPE cell apoptosis (18,19). Thus, lipofuscin is thought to be responsible for oxidative damage to RPE resulting in impaired metabolism and apoptosis characteristic of late AMD (15).

Mitochondria play a central role in aging and in the pathogenesis of age-related neurodegenerative diseases (25). Early studies have revealed several abnormalities in mitochondrial DNA (mtDNA) and subsequent disorders of respiratory enzyme complexes accompanied by reduced energy production, generation of excessive ROS and activation of the apoptosis pathway (26). However, recent studies suggest that mitochondrial dysfunctions may also include compositional and structural alterations in mitochondrial membranes (mtMEM) (27,28). Alterations in membrane lipid composition, such as decreases in cardiolipin content or changes in the ω -3/ ω -6 ratio, impair the electron transport chain and energy production (29), ion channel and Ca^{2+} homeostasis of the mitochondria and of the cell (30-33). These alterations can also impair carnitine-mediated lipid transport, mitochondrial lipid metabolism (34,35), cholesterol biosynthesis (36-39), activity of the pyruvate dehydrogenase complex (40,41) and, finally, cause activation of the apoptosis pathway (42). In the present study, detailed electron microscopy of human RPE cells from aged and AMD specimens are described. Based on morphometric data, we found that progressive deterioration of mtMEM with aging occurred in association with peroxisome proliferation and accumulation of lipofuscin in the RPE, and alterations in the RPE mtMEM and proliferation of peroxisomes were significantly more severe in AMD compared to normal aging.

Sensory innervation of the eye comes from the first branch of the trigeminal nerve and it plays an essential role in the physiology and pathophysiology of both the anterior and posterior segments of the eye (43,44). Sensory nerves regulate tear secretion either through the sensory arc of reflex tearing (45) or directly through releasing neuropeptides from sensory nerve endings (46). Both mechanisms are involved in the development of dry eye syndromes (47,48). Although the retina has no sensory innervations, substance P (SP)- and calcitonin gene-related peptide (CGRP)-containing amacrine and ganglion cells have been observed in the retina in various species including humans (49-51). Furthermore, several experimental studies have suggested that these neuropeptides are involved in various retinal diseases (52-54). Previously, an increase in SP and CGRP immunoreactivity was observed in the retina after electric stimulation of the trigeminal ganglion (55). However, the mechanism by which these neuropeptides are generated in the retina has yet to be elucidated. Capsaicin (CAP), a neurotoxic substance for polymodal C sensory nerve fibers, is widely used in experiments, either for sensory nerve stimulation or sensory nerve damage, depending on the dose applied (56). Animals exposed to CAP exhibit various corneal lesions, including a reduction in the number of corneal nerve fibers and disintegration of epithelial cells (57,58). In addition, changes in the physiology of the ocular surface, such as a reduction in tear fluid secretion, impairment of corneal epithelial barrier function and a delay in corneal epithelial wound healing, are apparent in such animals (59). These CAP-induced changes thus appear to be similar to

those characteristic of neurotrophic keratouveitis in humans (60). Degeneration of amacrine and ganglion cells has also been observed after CAP treatment (61). Pigment epithelium-derived factor (PEDF) has been isolated from the RPE and has also been found in the vitreous and the cornea (62). PEDF was originally identified as a neurotrophic factor (63). Subsequently, it was found to possess potent antiangiogenic activity (64,65). It has been shown that PEDF is essential for maintaining the avascularity of the cornea and vitreous (66,67) and that it influences CAP-induced neurotrophic keratouveitis. We confirm that retrobulbar administration of PEDF may attenuate the effect of CAP on tear secretion, keratouveitis and the retina.

Materials and methods

Sixty-five human eyes (age range 2-87 years) were selected for these electron microscopic studies. All experiments were conducted in accordance with the Declaration of Helsinki (1964) and with the understanding and consent of the human subjects. The responsible ethics committee approved the experiments. Three eyes, aged 2, 7 and 27 years, were used only for qualitative analysis of age-related changes. Thirty-one of the eyes were affected by early AMD (ages from 42 to 87 years, mean age 70.9 years; 20 female and 11 male) and 31 non-affected eyes were used for age- and gender-matched controls for both the qualitative and quantitative morphometric studies. The selection criteria for early AMD was based on the presence of drusen and/or basement membrane thickening of the RPE, while in the controls no drusen or basement membrane thickening of RPE was observed by electron microscopy. Late forms of AMD (geographic atrophy and/or choroidal neovascularization) were excluded from these studies. All of the human eyes had been surgically removed as a result of malignant tumors or severe ocular trauma, neither of which affected the posterior pole of the eyeball.

One hundred and forty-four 4-week-old Sprague-Dawley rats, weighing ~200 g and of both sexes, maintained in standard laboratory conditions at 22°C temperature, 60% humidity and a 12-h light/dark cycle, were divided into 6 groups of 24 rats each. Care and treatment of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, avoiding animal suffering at each stage of the experiments. Rats of the first group (CAP) were given a single retrobulbar injection of CAP 50 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) in a procedure described elsewhere. Rats in the second (CAP+PEDF 3.2 from Day 0) and third (CAP+PEDF 6.4 from 0) groups received the same injection of CAP plus daily retrobulbar injections of 3.2 or 6.4 $\mu\text{g/kg}$ PEDF starting from Day 0 (BioProducts, Middletown, MD, USA), respectively. Rats in the fourth (CAP+PEDF 3.2 from Day 14) and fifth (CAP+PEDF 6.4 from Day 14) group also received the same CAP plus daily retrobulbar injection of 3.2 or 6.4 $\mu\text{g/kg}$ PEDF starting at Day 14 after CAP challenge. The sixth (control) group (C) received the vehicle solution alone. One randomly selected eye of each rat was used and the untreated fellow eye was used to evaluate eventual systemic effects. PEDF treatment was repeated daily until the end of the study.

Electron microscopy. Small pieces of the human retina and choroid were dissected at the posterior pole <2 min after removal of the eyeball and fixed at 4°C in 2% buffered glutaraldehyde for 2 h and postfixed in 2% osmium tetroxide for another 2 h. The postfixation with osmium tetroxide demonstrates lipid peroxides as effectively as tetramethylbenzidine but avoids incubation, which might damage membrane structures (68). The specimens were dehydrated, embedded in Araldite, sectioned with a Reichert ultramicrotome (Reichert, Vienna, Austria), contrasted with lead citrate and uranyl acetate and studied with a Zeiss 109 electron microscope.

Small pieces (1x1 mm) of the rat cornea were dissected and fixed in buffered 2% glutaraldehyde for 2 h, postfixed in 2% osmium tetroxide for another 2 h, dehydrated and embedded in Araldite. Ultrathin sections were cut with an ultramicrotome (Reichert), contrasted with uranyl acetate and lead citrate and studied with an electron microscope. After prefixation, rat tissue samples were oriented and the exposed surface was coated with gold-carbon vapor and examined with an electron microscope equipped with a high-resolution scanning device used for photography (EM Asid JEM-100B; JEOL, Tokyo, Japan).

Light microscopy. Six rats from each group were sacrificed using carbon dioxide at Days 7, 14, 28 and 42. The eyes were enucleated and fixed in Karnovsky's solution for 48 h, dehydrated and embedded in paraffin, and the 6- μ m sections were colored with H&E and Masson's trichrome staining for light microscopy. In addition, myeloid leukocytes labeled with chloroacetate-esterase were counted in the H&E-stained sections under light microscopy. Chloroacetate-esterase specifically identifies cells of the granulocyte lineage, from the early promyelocyte stage to mature neutrophils. The number of leukocytes was counted in the anterior chamber, posterior chamber, peripheral retina and peripheral vitreous at the same microscopic magnification.

Morphometry. In the parafoveal area of the macula, the total number of mitochondria, number of lipofuscin granules and number of peroxisomes were counted at a magnification of x8,000. The number of well-defined mitochondrial cristae and the area of the mitochondria and lipofuscin, were measured in 6-8 RPE cells/specimen at a magnification of x20,000. Each organelle was outlined and the area was determined using the NIH Image J program. Morphometric analysis was performed by two experienced observers, and the questionable cases were re-evaluated by the senior researcher.

Tear secretion. Tear fluid secretion was measured without topical anesthesia by a modified version of the Schirmer's test. Schirmer strips cut to dimensions of 20x1 mm were inserted below the lower eyelid of the test animal for 1 min. The wet length of the strip was measured to an accuracy of ± 0.5 mm. The Schirmer's test was performed at Day 1 (before any treatment) and at Days 7, 14, 28 and 42 after CAP injection.

Statistical analysis. Data are expressed as means \pm SD and were analyzed using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). In the first experimental model to measure the relation between age and the evaluated parameters, linear

regression analysis was applied for determining the P-value and Pearson correlation coefficient, r. We used 2-sided comparisons to detect any difference between linear regression results of the aged and AMD groups. In all cases $P < 0.05$ was considered to indicate a statistically significant result.

In the second experimental model, statistical analysis was performed with repeated-measures ANOVA using the Dunnett's multiple-comparison test for results in the 3 groups. After Bonferroni adjustment, the significance level was set at $P < 0.01$.

Results

Concerning age-related changes of the RPE and Bruch's membrane, electron microscopy showed very marked differences between young and aged human specimens (Fig. 1A and B). Mitochondria in young RPE were numerous, mostly bacillus-like shaped and oriented parallel to the apical-basal axis (Fig. 1A). They were typically rich in well-preserved cristae. In normal aged eyes, mitochondria of the RPE clearly decreased in number (Fig. 1B), were variable in size and were usually oval-shaped without any preferential orientation. In some instances, there was disorganization of cristae, ranging from focal to complete loss, in association with decreased electron density of the matrix. Mitochondria of the RPE in AMD eyes (Fig. 2B) appeared to decrease in number and size when compared to the normal aging eyes (Fig. 2A). They were often round or oval, with focal or even complete loss of cristae and associated with more extensive decreases in matrix density (Fig. 3). In some instances, either bleb formation or interruption of the mitochondrial internal and external membranes was observed. These may be considered pre-apoptotic alterations. All of these mitochondrial alterations were apparently more marked and more extensive in AMD compared to normal aging. Bruch's membrane fine structure was clearly visible in young eyes (Fig. 1A) and consisted of a core elastic layer, outer and inner collagenous layers, basement membranes of the RPE and choriocapillaries. During normal aging, there was an increase in electron density of Bruch's membrane (Fig. 1B). Moreover, both inner and outer collagenous layers contained electron-dense granular and vesicular material. Bruch's membrane showed characteristic differences in AMD compared to normal aged eyes. In addition to the age-related increase in thickness and electron density of collagenous layers, AMD specimens usually showed multiplex, focal thickenings of the inner collagenous layer known as hard or soft drusen. Apart from drusen, thickenings of the RPE basement membrane due to basal laminar deposits were also present. In most cases they were focal, wart-like depositions of filamentous material in which several electron-dense areas, possibly composed of lipids, were present. Cytoplasmic processes of the RPE invading this excessive basement membrane material were frequently observed.

Peroxisomes in the RPE were 0.1-0.3 μ m in diameter, smaller than that in other cells (Fig. 1A and B). Some of these peroxisomes had electron-dense cores while others had a granular appearance. They were usually localized in the basal region of the cytoplasm and occasionally next to the basal and basolateral cell membrane. In normal aged

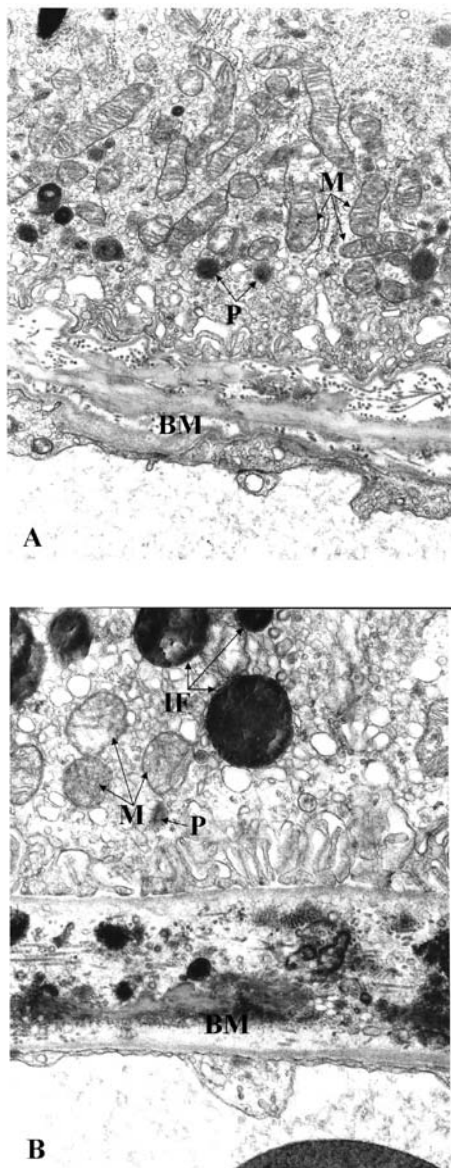


Figure 1. Representative electron micrographs of the RPE and Bruch's membrane. (A) Young RPE cells contained numerous bacillus-like mitochondria (M) with long axes oriented from the apical to the basal surfaces of the RPE. They were parallel to one another and closely packed, filling a large portion of the cytoplasm. The mitochondrial cristae were well preserved and the matrix was homogeneously electron-dense. The cytoplasm contained several rough-surface endoplasmic reticulum along with numerous small vesicles, probably microsomes. Several peroxisomes (P) appeared as small, round, electron-dense organelles. No lipofuscin granules were noted. There were numerous basal infoldings of the plasma membrane. Bruch's membrane (BM) ultrastructure was clearly visible and included an elastic core layer with some interruptions, inner and outer collagenous layers, basement membrane of the RPE and choriocapillaries. Two-year-old male; magnification, x25,000. (B) Aged RPE cells had mitochondria (M) that showed various degrees of membrane disorganization. Mostly, there were focal losses of cristae accompanied by decreased electron density of the matrices. Numerous lipofuscin (IF) and melano-lipofuscin granules were present in the cytoplasm of the RPE. Peroxisomes (P) of various density, shape and size were distributed randomly in the cytoplasm of the RPE cells, even among the lipofuscin granules. Electron-dense homogeneous or granular material was present in Bruch's membrane (BM). Sixty-five-year-old female; magnification, x28,000.

eyes, peroxisomes were more numerous and more variable in size, electron-density and distribution than in young eyes. Furthermore, in young eyes they were dispersed

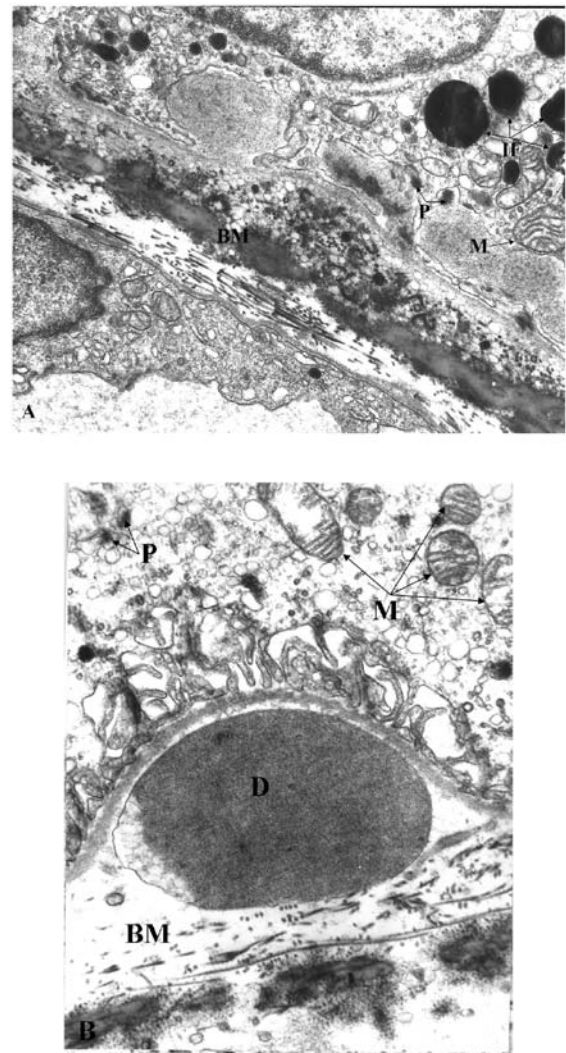


Figure 2. Comparison of the RPE in aged and AMD eyes. (A) In normal aging, most of the RPE mitochondria (M) had typical morphology, although several had focal loss of cristae and decreased electron density of the matrix. This micrograph also shows the thin basement membranes of the RPE and choriocapillaries, as well as interruptions of the elastic layer and deposition of amorphous materials in Bruch's membrane (BM). Some small round deposits were present in the collagenous layers of Bruch's membrane. Eighty-four-year-old female; The eye was removed due to malignant tumor, and the specimen came from the unaffected area; magnification, x26,000. (B) In AMD, advanced mitochondrial alterations occurred in the RPE. Most mitochondria (M) had severe disorganization of membranes that varied from focal to complete loss of cristae and decreased electron density of the matrix. Several peroxisomes (P) were randomly distributed in the basal cytoplasm of the RPE. Focal thickenings of the RPE basement membrane and a drusen (D) were also present in Bruch's membrane (BM). Several cytoplasmic processes protruded into the thickened basement membrane. Seventy-four-year-old female; the eye was removed due to a perforating injury; magnification, x32,000.

randomly in the cytoplasm of the RPE, while in aged eyes they formed small groups containing 4-5 peroxisomes. The distribution of peroxisomes in AMD eyes was highly variable within each RPE cell. Occasionally, they were located in the apical cytoplasm among the lipofuscin granules. More rarely, peroxisomes were in close topographic correlation with mitochondria and the nucleus of the RPE cells. In osmium-fixed specimens the peroxisomes varied in electron density. Lipofuscin granules and residual bodies were exceptionally rare at an early age, but they clearly increased

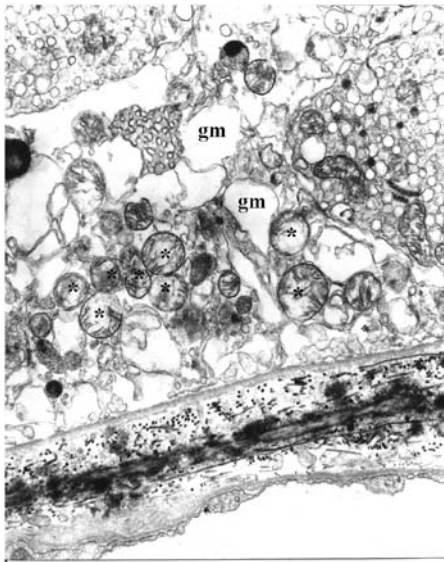


Figure 3. Representative electron micrograph of mitochondrial alterations in AMD. Extensive loss of cristae and matrix density occurred in most mitochondria (*). Several had complete loss of internal structure and appeared as 'ghost mitochondria' (gm). Some peroxisomes were adjacent to the mitochondria. Seventy-one-year-old female; magnification, x22,000.

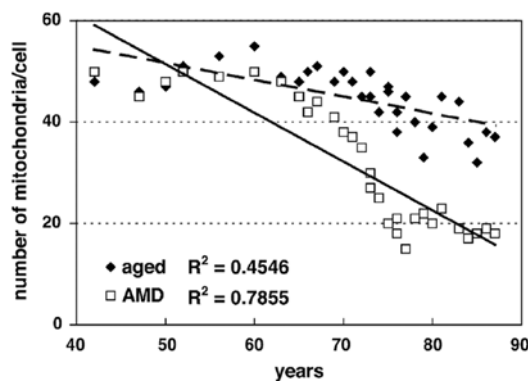


Figure 4. In both aged and AMD groups, there were significant decreases in the number of mitochondria with age ($P<0.001$). In addition, the rate of loss in the AMD group was significantly greater than that in the normal aging group ($P=0.038$).

in number and size with time (Fig. 1B). In aged adults they were abundantly distributed in the cytoplasm of the RPE. Numerous melanolipofuscin granules were also present, formed by fusion of melanosomes and lipofuscin. These organelles were located in the apical half of the RPE in normal aged eyes. Lipofuscin granules and residual bodies in AMD specimens presented a morphology and distribution similar to control eyes.

Morphometric analysis of the electron micrographs quantified the mitochondrial and peroxisomal alterations. The total number of mitochondria decreased significantly in both aged ($R^2=0.455$; $P<0.001$) and AMD ($R^2=0.778$; $P<0.001$) groups (Fig. 4). The decrease in the AMD group was more severe and the difference was statistically significant ($P=0.038$). The number of mitochondria at age 75 in the AMD group was equivalent to that at age 85 in the control group. These

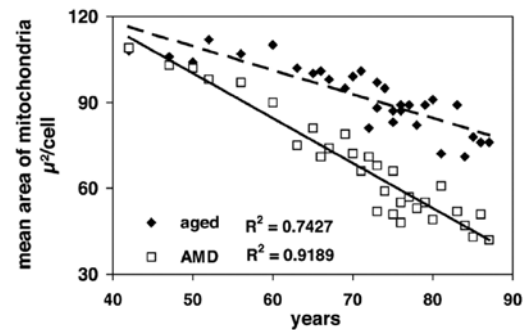


Figure 5. Both aged and AMD groups had significant decreases in the area of mitochondria with age ($P<0.001$). This decrease was significantly more marked in AMD patients compared to age-matched controls ($P=0.019$).

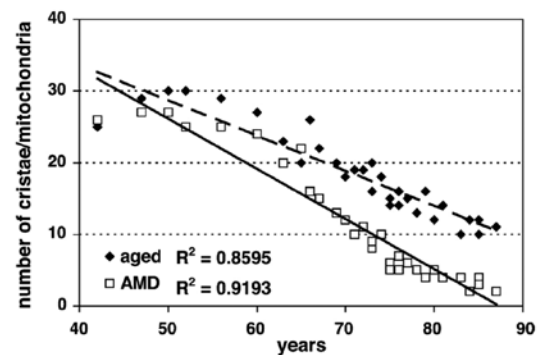


Figure 6. Changes in the number of mitochondrial cristae. Both aged and AMD mitochondria showed significant loss of cristae ($P<0.001$). Although the disorganization of mitochondrial cristae was more extensive in the AMD group compared to age-matched controls, the difference between them was not statistically significant ($P=0.28$).

findings suggest that with age the number of mitochondria decreased more rapidly in AMD compared to normal aged patients.

The area of the mitochondria also decreased significantly with age in both control ($R^2=0.743$; $P<0.001$) and AMD ($R^2=0.919$; $P<0.001$) groups (Fig. 5). The decrease in the AMD group was again more severe and the difference between the control and AMD groups was statistically significant ($P=0.019$). The area of mitochondria at 85 years in the control group was similar to that of 70 years in the AMD patients; the decrease in area of mitochondria developing 15 years earlier in the AMD patients.

The number of well-defined mitochondrial cristae was also counted and it showed a significant decrease in both the control ($R^2=0.861$; $P<0.001$) and AMD ($R^2=0.918$; $P<0.001$) groups (Fig. 6). However, the difference between controls and AMD was not significant ($P=0.28$). Comparison of data from the control group at 85 years showed again that the same loss of well-defined mitochondrial cristae developed 15 years earlier in AMD. The area of reduced matrix density corresponded to the loss of cristae (data not shown). Morphometric analysis showed a significant increase in the number of peroxisomes in both the control ($R^2=0.207$; $P<0.001$) and AMD ($R^2=0.608$; $P<0.001$) groups (Fig. 7). Moreover, the difference between the controls and AMD was statistically

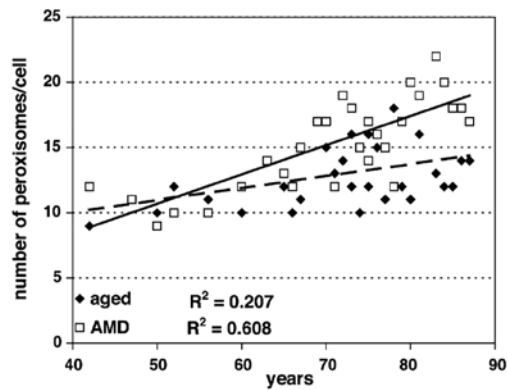


Figure 7. The number of peroxisomes increased with age in the aged ($P<0.001$) and AMD ($P<0.001$) groups. Furthermore, the increase was significantly greater in the AMD than in the age-matched controls ($P=0.044$).

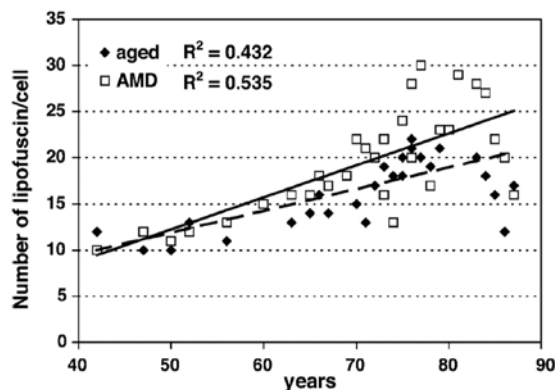


Figure 8. The number of lipofuscin granules increased significantly with age in the aged ($P=0.01$) and AMD ($P<0.001$) groups. The difference between the groups was not significant ($P=0.61$).

significant ($P=0.044$). Comparison of data from the normal aged group at 85 years showed the same levels ~10 years earlier in the AMD group. There was a significant increase in lipofuscin granules in both the control ($R^2=0.432$; $P<0.001$) and AMD ($R^2=0.535$; $P<0.001$) groups (Fig. 8). However, the difference between the two groups was not statistically significant ($P=0.61$). Comparison of data from the normal aged group at 85 years showed that the same increase in the number of lipofuscin granules developed ~15 years earlier in the AMD group. Our study did not reveal any substantial differences between females and males in both the aged and AMD groups.

In the second experimental model, we evaluated the tear fluid secretion by Schirmer's test in animals treated by retrobulbar injection of CAP and PEDF. CAP treatment resulted in a statistically significant decrease in tear secretion at Days 1 (2.50 ± 0.55), 7 (3.17 ± 0.41), 14 (3.50 ± 0.55), 28 (3.50 ± 0.55) and 42 (3.67 ± 0.52) compared with the control values (6.83 ± 1.33), (6.50 ± 1.38), (6.67 ± 0.52), (6.67 ± 0.52) and (7.17 ± 0.98), respectively. Treatment with 3.2 or 6.4 $\mu\text{g/kg}$ PEDF from Day 0 attenuated the effect of CAP at Days 28 and 42 ($P<0.001$) only, and a significantly decreased tear secretion was measured in group CAP+PEDF 3.2 at Days 1 (2.67 ± 0.52), 7 (3.33 ± 0.82), 14 (4.17 ± 0.75), 28 (4.83 ± 0.41) and 42 (5.33 ± 0.52) and in group

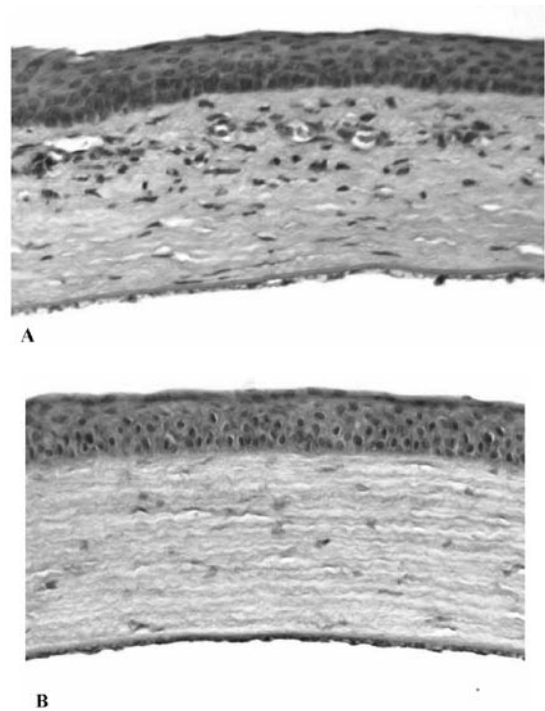


Figure 9. Light microscopy of CAP-induced keratouveitis. (A) H&E staining. The cornea of the CAP-treated eye showed a thin and irregular epithelium, superficial scar tissue and neovascularization. Magnification, $\times 200$. (B) H&E staining. The cornea of the CAP + PEDF 6.4 group had a normal appearance (Day 42). Magnification, $\times 200$.

CAP+PEDF 6.4 at Days 1 (2.50 ± 0.55), 7 (3.83 ± 0.41), 14 (4.33 ± 0.52) and 28 (5.33 ± 0.52) compared with the controls. PEDF treatment initiated at Day 14 also attenuated the effect of CAP pretreatment on tear secretion, but these effects were not significant at any time point.

A single retrobulbar injection of CAP into young rats caused a clinically marked inflammation of the anterior segment progressing from slight punctate vacuolization in the epithelium at the second or third days to diffuse edematous opacities and neovascularization in the stroma, at the third or fourth week, persisting at least for 6 weeks. These alterations in the cornea showed continuous progression by the end of follow-up. In contrast, by administration of PEDF, both corneal opacities and scar formation were prevented and the cornea became completely transparent at the end of the treatment. The most prominent histopathologic feature of the affected corneas was a marked disorganization of the epithelium, followed by marked polymorphonuclear leukocyte influx as well as by edema and disorganization of the corneal stroma with degeneration and loss of the central epithelium. These alterations were accompanied by fibrinous and cellular exudation into the anterior chamber (Fig. 9A). Interstitial edema in the corneal epithelium was particularly evident, even in the basal and intermediate layers of the epithelium of CAP-treated animals. There was extensive corneal neovascularization occurring approximately on Day 14. Myeloid cell infiltration was also evident within the angle, the anterior chamber and the iris. Treatment with PEDF prevented and/or significantly attenuated corneal epithelial and stromal damage in a dose-dependent manner (Fig. 9B). These data are provided in detail in Table I.

Table I. Number of myeloid cells in the eyes at different time points of the study.

Group	Localization	Day 7	Day 14	Day 28	Day 42
Control	AC	0.17±0.40	0.33±0.51	0.33±0.51	0.5±0.55
	PC	0.16±0.41	0.33±0.51	0.33±0.51	0.5±0.55
	Vitreous	0.16±0.41	0.33±0.51	0.33±0.51	0.5±0.55
	Retina	0.16±0.41	0.33±0.51	0.33±0.51	0.5±0.55
CAP-treated	AC	10.5±2.16 ^a	12.5±2.95 ^a	17.83±3.92 ^a	22.16±2.14 ^a
	PC	9.17±0.75 ^a	12.5±1.38 ^a	17.83±1.72 ^a	21.5±2.07 ^a
	Vitreous	2.66±0.52 ^a	4.67±1.21 ^a	7.67±0.82 ^a	9.33±1.75 ^a
	Retina	5.83±1.47 ^a	9.83±2.14 ^a	15±2.61 ^a	19±2.83 ^a
CAP+3.2 µg PEDF from Day 0	AC	7±0.89 ^{a,b}	9±0.89 ^{a,b}	5.83±1.16 ^{a,b}	5.16±0.75 ^{a,b}
	PC	6.5±0.5 ^{a,b}	7.67±0.82 ^{a,b}	5.5±0.83 ^{a,b}	4.5±0.55 ^{a,b}
	Vitreous	2.5±0.55 ^a	2.83±0.41 ^{a,b}	3.5±1.22 ^{a,b}	1.83±0.41 ^b
	Retina	3±0.63 ^{a,b}	3.67±1.03 ^{a,b}	4.33±0.82 ^{a,b}	2.67±1.03 ^b
CAP+6.4 µg PEDF from Day 0	AC	3.83±0.41 ^{a,b}	4.33±0.52 ^{a,b}	4.16±1.47 ^{a,b}	2.33±1.51 ^b
	PC	3.83±0.41 ^{a,b}	3.83±0.75 ^{a,b}	3.5±0.83 ^{a,b}	2±1.26 ^b
	Vitreous	1.66±0.82 ^a	2.66±0.52 ^{a,b}	2±1.26 ^b	0.33±0.52 ^b
	Retina	3.67±0.52 ^{a,b}	2.67±0.51 ^{a,b}	2.5±1.04 ^b	1±0.89 ^b
CAP+3.2 µg PEDF from Day 14	AC	10.5±0.89 ^a	12.8±0.89 ^a	11.4±0.89 ^{a,b}	10.0±0.89 ^{a,b}
	PC	9.8±1.03 ^a	12.6±0.98 ^a	12±0.89 ^{a,b}	10.3±0.83 ^{a,b}
	Vitreous	3.1±0.75 ^a	5.3±0.83 ^a	5±0.83 ^{a,b}	4.8±0.89 ^{a,b}
	Retina	6.8±0.89 ^a	10.6±0.89 ^a	9.4±0.98 ^{a,b}	8.5±0.98 ^{a,b}
CAP+6.4 µg PEDF from Day 14	AC	11±0.98 ^a	13.1±0.89 ^a	11.6±0.89 ^{a,b}	9.7±0.89 ^{a,b}
	PC	9.9±0.89 ^a	12.4±1.14 ^a	11.8±0.89 ^{a,b}	9.3±0.82 ^{a,b}
	Vitreous	4±0.83 ^a	6.0±0.82 ^a	5.1±0.75 ^{a,b}	4.3±0.83 ^{a,b}
	Retina	7.2±0.89 ^a	11.2±0.89 ^a	9.7±0.98 ^{a,b}	7.6±0.89 ^{a,b}

AC, anterior chamber; PC, posterior chamber; ^aP<0.001 compared with the control group; ^bP<0.001 compared with the CAP group at the given time point.

Discussion

Our electron microscopic study demonstrated that mitochondria of the RPE undergo significant morphological changes with age as a result of marked decreases in the number and area of mitochondria, significantly more severe in ADM compared to age-matched controls. These changes include a partial to complete loss of cristae and decrease in the density of the mitochondrial matrix in both normal aging and AMD groups. To our knowledge, this is the first study to demonstrate alterations in mtMEM related to age and AMD in human RPE. It also revealed significant differences between normal aging and AMD. Ultrastructural and morphometric studies showed similar alterations in mtMEM in certain age-related human diseases, such as Alzheimer's disease (69), in the skeletal muscle of patients affected by type 2 diabetes or obesity (70) and in heart failure with chronic obstructive pulmonary disease (71), as well as in schizophrenia (72). Our findings suggest that RPE in aging and AMD should be included in that list. These data provide morphological support to the concept that mtMEM and subsequent mitochondrial dysfunctions may play a crucial role in the development of retinal degeneration, in particular of AMD (16,73). Our data suggest

that loss of mitochondrial structure is one of the differences between normal aging and AMD. Photoreceptors and RPE have an intimate morphologic and functional partnership in order to maintain adequate metabolic support for survival, excitability and turnover of photoreceptor cells. Mitochondrial β -oxidation, which is present in the RPE (74), is considered to be the major pathway that metabolizes fatty acids as a primary source of energy production (75,76). Since the RPE is involved in the light-induced retinoid cycle (77,78), one of the possible consequences of mitochondrial dysfunction in AMD may manifest as a disorder of light-induced retinoid recycle. Mitochondrial alterations were also accompanied by proliferation of peroxisomes. The morphometrical study showed that an age-related increase in peroxisome number in AMD specimens was significantly greater than the number in age-matched controls. Peroxisomes are membrane-bound organelles that play an essential role in lipid metabolism. They shorten the very-long-chain fatty acids, and the resulting long-chain fatty acids preferentially move back to the endoplasmic reticulum where they are used for membrane lipid synthesis (79), or they are then handed over to mitochondria for completion of oxidation (80). Intracellular elevation of naturally occurring fatty acids and eicosanoids activates

peroxisome-proliferator-activated receptors (PPARs) resulting in peroxisome proliferation and activation of lipid metabolism (81). Activation of PPAR- α upregulates genes of lipid catabolism, while activation of PPAR- γ upregulates genes of lipogenesis (82,83). In a previous study (84), PPAR- γ ligands inhibited vascular endothelial growth factor-induced choroidal angiogenesis *in vitro* and choroidal neovascularization *in vivo*. This suggests the potential involvement of PPAR- γ in AMD, at least in the development of the late form of this disease. The increased number of peroxisomes in aging and AMD may be a morphologic manifestation for activation of an alternative pathway for lipid degradation. This hypothesis is supported by observations that induction of PPAR- α was associated with a strong stimulation of the enzymes involved in peroxisomal β -oxidation (85,86). However, only PPAR- γ has been detected in human RPE (84). Accumulation of partially metabolized lipids such as lipofuscin and lipid peroxides in RPE may be another possible consequence of mitochondrial dysfunction (20,87). Lipofuscin may be responsible for oxidative damage to the RPE that results in impaired metabolism and apoptosis characteristic of late AMD (15). Studies suggest that lipofuscin is a manifestation of the balance between production and elimination of partially metabolized substances, mostly lipid peroxide-containing materials (88,89). Thus, the age-related accumulation of intracellular lipofuscin is an indicator for impairment of lipid degradation processes (90). A previous autofluorescent study (91) showed a higher lipofuscin content in AMD compared to age-matched controls. Lipofuscin and other metabolized lipids containing ROS may certainly compromise RPE functions. However, the exact role of lipofuscin in AMD remains to be elucidated. Alterations in mtMEM influence the carnitine system located in the mitochondrial outer and inner membrane resulting in impaired lipid metabolism (35). mtMEM play an essential role in cholesterol metabolism in the liver and in several extrahepatic cells including macrophages (38), which have several features in common with RPE. A peripheral type benzodiazepine receptor, a channel-forming mitochondrial protein, is involved in the regulation of cholesterol transport from the outer to the inner mtMEM. This is the rate-determining step in steroid biosynthesis. The lipid composition of mtMEM might be involved directly in ion channel regulation (31). A decrease in mtMEM potential occurs in a variety of aging cell types from several mammalian species (32,33). When mitochondria are subjected to oxidative stress and relatively high Ca^{2+} , they may undergo a permeability transition in which the inner membrane becomes freely permeable to low-molecular-weight substances resulting in impairment of all mitochondrial functions (30). Mitochondrial ion channels are critically involved in apoptotic changes in mitochondria (42). In addition to the well-documented changes in mtDNA, our data suggest that alterations in mtMEM also play a crucial role in the development of mitochondrial dysfunctions in AMD and, possibly, in other age-related diseases (92). This has an immediate clinical relevance as shown by several *in vitro* and *in vivo* studies. In fact, mtMEM could be a target for treatment of mitochondrial dysfunctions. *In vitro* modification of mtMEM composition by ω -3 fatty acids and addition of carnitine or acetyl-L-carnitine resulted in a subsequent improvement in mitochondrial lipid metabolism (93). An *in vivo* study showed that dietary ω -3

fatty acids directly increase membrane ω -3/ ω -6 ratio, restore mtMEM cardiolipin content and membrane potential, as well as subsequently restore altered mitochondrial Ca^{2+} flux and Ca^{2+} -dependent processes such as activity of pyruvate dehydrogenase complex (94).

Furthermore, research demonstrated that a retrobulbar injection of CAP into young rats resulted in decreased tear secretion and neurotrophic keratouveitis characterized by epithelial alteration, stromal edema and scar formation accompanied by neovascularization of the cornea (60). In contrast to this study, we observed leukocyte infiltration in the posterior chamber, peripheral retina and vitreous body. These CAP-induced alterations were attenuated in a dose-dependent manner by retrobulbar injection of PEDF. CAP exerts its effects through binding to transient receptor potential vanilloid type 1 (TRPV1), which is a Ca^{2+} -permeable ion channel. CAP may act on TRPV1 receptors of non-neuronal cells or sensory nerves of the cornea, conjunctiva, lacrimal glands, ciliary body and choroids. The activation of TRPV1 in the sensory nerve endings induces the release of the proinflammatory neuropeptides SP and CGRP, resulting in neurogenic inflammation, that may have been a leading contributing factor to the CAP-induced keratouveitis in our model. Non-neuronal cells include epithelial cells (keratinocytes, urothelium, gastric epithelial cells, enterocytes and pneumocytes), vascular endothelium and cells of the immune system as well as smooth muscle cells, fibroblasts and hepatocytes (95). PEDF accelerated the recovery of tear secretion and prevented neurotrophic keratouveitis and peripheral vitreoretinal inflammation by neurotrophic and antiangiogenic mechanisms. *In vitro* studies have shown that neuronal growth factors exert their effects through modulating TRPV1 expression and activity of sensory nerve cells (96). Recently, topical treatment with nerve growth factor (NGF) was also shown to restore corneal integrity in humans with corneal neurotrophic ulcers or keratitis (97). Moreover, there is accumulating evidence that in normal conditions there may be a balance between the release of PEDF and vascular endothelial growth factor (VEGF) (98). A decrease in the levels of PEDF or an increase in VEGF may be responsible for neovascularization in the exudative form of age-related macular degeneration (AMD), diabetic retinopathy and ischemia-induced retinal neovascularization (99). These findings suggest a certain antagonism between PEDF and VEGF (98). Controversial studies have shown direct neurotrophic effects of VEGF similar to nerve growth factors suggesting a synergy between them in providing neuroprotection (100-102). Further studies are certainly needed to reveal the molecular mechanism of the association between PEDF and VEGF in both neuroprotection and angiogenesis.

In conclusion, our study on restoration of mitochondrial function are certainly very promising as they open up a new therapeutic approach to AMD. However, the correlation between mitochondrial dysfunction and Bruch's membrane alterations remains to be elucidated through ongoing studies. Clinical studies confirmed that a combination of acetyl-L-carnitine, ω -3 fatty acids and Coenzyme Q10, after an initial improvement, stabilized several visual functions in early AMD (103). However, further studies are also necessary to reveal how the clinically known risk factors of AMD are

different between normal aging and AMD and why alterations of the RPE common to both normal aging and AMD groups occur at a younger age in AMD. This is also the first experimental study to suggest a neuroprotective and antiangiogenic effect of PEDF in CAP-induced keratouveitis. Although the involvement of TRPV1 is presumed, the molecular mechanisms of PEDF remain to be elucidated. PEDF is certainly related to neuroprotection and angiogenesis, but its role in the pathophysiology of the ocular compartment remains somewhat unclear.

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