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Egg yolk improves lipid profile, lipid peroxidation and retinal abnormalities in a murine model of genetic hypercholesterolemia☆

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Abstract

Carotenoids are believed to inhibit oxidative stress. We investigated the protective effect of lutein and egg yolk supplementation on systemic and retinal alterations in apolipoprotein E-deficient (apoE−/−) mice, an experimental model of hypercholesterolemia and cardiovascular disease. Three-month-old wild-type and apoE−/− mice received one of the following: vehicle, lutein (0.09 mg/kg per day) or egg yolk (0.8 g/kg per day), by gastroesophageal cannula for 3 months. Total cholesterol (TC), triacylglycerol (TG) and lipid peroxidation (TBARS) were measured in plasma. TBARS levels were also determined in retinal homogenates. Ultrastructural morphology was analyzed by electron microscopy. ApoE−/− mice, with increased TC and TG concentrations, had higher systemic ($P<.05$) and retinal ($P<.01$) levels of lipid peroxidation than wild-type strains. Electron microscopy showed ultrastructural alterations (basal laminar deposits, open intercellular junctions, increased cytoplasmic vacuoles) in the retinas from apoE−/− mice. Egg yolk significantly reduced plasma TG ($P<.05$) and, without changes in TC, decreased plasma lipid peroxidation ($P<.05$). Lutein supplementation marginally affected the parameters. Less severe retinal ultrastructural alterations were observed in apoE−/− mice receiving either egg yolk or lutein. In the apoE−/− mouse model, egg yolk improved the lipid profile and reduced systemic lipid peroxidation ($P<.05$). While lutein and egg yolk did not seem to reduce retinal lipid peroxidation, a reduction in retinal ultrastructural alterations was observed.

Keywords: Egg yolk; Lipid profile; Lipid peroxidation; Hypercholesterolemia; Lutein

1. Introduction

Dietary fat and cholesterol are related to an increased incidence of coronary heart disease. Oxidative stress plays an important role in the initiation and progression of atherosclerosis by stimulating inflammation and promoting cytokine production [1,2]. Hypercholesterolemia and a high-fat diet also have been proposed as possible risk factors for retinal pathologies such as age-related macular degeneration (ARMD), the most important cause of visual loss among the elderly [3,4]. The initial stage of ARMD is characterized by accumulation of lipid-rich deposits under the retinal pigment epithelium (RPE) and Bruch's membrane (BrM). Degenerative changes in the RPE and photoreceptor cells are early events in ARMD [5]. Moreover, animal studies have shown that dietetic or genetic hypercholesterolemia induces formation of subretinal deposits and retinal alterations similar to those in the aging human retina [6–9].

A potential role of antioxidant vitamins in the prevention of cardiovascular disease has been proposed, based on the essential role of oxidative stress in atherosclerosis. A number of studies in different animal models have considered the potential for dietary antioxidants to prevent cardiovascular and retinal pathologies, and most of the experimental evidence substantiates this hypothesis [10–13]. However, to date there is no clear evidence from

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clinical trials that dietary intake of antioxidants or vitamin supplementation with antioxidants reduces the risk of cardiovascular disease.

In contrast to the lack of beneficial effects of antioxidant vitamins in reducing atherosclerotic risk, recent clinical studies have shown that antioxidant treatment stops progression of some retinal processes [14,15]. Lutein and zeaxanthin were recently reported to be in the photoreceptor rod outer segments where there are large amounts of polyunsaturated fatty acids with high vulnerability to oxidation, suggesting that these carotenoids are antioxidants that may prevent ARMD [16,17].

Lutein and zeaxanthin are carotenoids present in the retina, where they bind to the retinal protein tubulin and protect visual function. Zeaxanthin is specifically concentrated at the macula, whereas lutein is distributed throughout the retina [17–20]. Although lutein and zeaxanthin can be obtained from certain vegetables, such as spinach, corn and pumpkin, egg yolk is a unique animal source and provides the highest bioavailability [21]. Carotenoids in egg yolk are in a digestible lipid matrix consisting of cholesterol, phospholipids and triacylglycerol (TG), which is thought to be optimal for dietary carotenoid absorption [22].

Recently, using a porcine model of hypercholesterolemia, we showed that increased retinal lipid peroxidation, anion superoxide and nitric oxide (NO) metabolite production decreased to control levels following supplementation with vitamins C and E. Morphologic and ultrastructural alterations in the RPE also have been documented in apolipoprotein E-deficient mouse (apoE−/−), a murine model of hypercholesterolemia and atherosclerosis characterized by increased oxidative stress [23]. These observations suggest a pathophysiologic link between abnormal lipid metabolism, oxidative stress and subretinal deposits [6,24].

Based on its proposed protective role against oxidation, we hypothesized that lutein could prevent systemic and retinal biochemical changes associated with hypercholesterolemia. We investigated the effect of lutein supplementation on oxidative stress. We also assessed whether egg yolk, as the most available dietary source of lutein, exhibited effects similar to those expected for lutein.

2. Materials and methods

2.1. Animals and experimental design

Thirty 3-month-old male C57BL/6J mice and 30 homozygous apoE−/− mice were used for this study. Progenitor couples were obtained from the Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium. All experimental procedures were approved by the Animal Research Ethics Committee of the University of Navarra.

Animals were randomly divided into six groups ($n=10$ each), fed a standard rodent chow (9605/8, Harlan Teklad TRM, Madison, WI, USA) and water ad libitum for 90 days, and housed in cages containing five mice each in a temperature-controlled room (20–22°C) with a 12-h light/dark cycle. The six study groups were as follows: wild-type controls (WT-C) that received vehicle; wild-type plus egg (WT-E), egg yolk 0.8 g/kg per day (comparable to two egg yolks daily for a human adult, providing 0.013 mg/kg per day of lutein); wild-type plus lutein (WT-L), lutein 0.09 mg/kg per day; apoE−/− control (AE-C), vehicle; apoE−/− plus egg yolk (AE-E), egg yolk 0.8 g/kg per day; and apoE−/− plus lutein (AE-L), lutein 0.09 mg/kg per day.

The treatments were emulsified in a mixture of water/soybean oil/Tween-80 (1:1:0.05; v/v/v) administered daily by gastroesophageal cannula for 3 months (100 μl). Purified lutein was kindly provided by Dr. Christine Gartner (Cognis, Germany).

2.2. Plasma separation and retinal homogenate preparation

At the end of treatment, the animals were euthanized with carbon dioxide, and blood samples were collected by cardiac puncture. EDTA plasma was obtained by centrifugation (4°C), frozen in liquid nitrogen and stored at −80°C.

Immediately after the blood samples were collected, the eyes were enucleated and transferred to a saline solution (pH 7.4). The retinas were rapidly dissected by making a small incision with a scalpel 1 mm behind the limbus; the incision was extended 360° using fine ophthalmic scissors.

The cornea, iris and lens were removed, and the remaining eyecup, lined with retina, was transferred to a 100-μM butylated-hydroxytoluene solution in saline, frozen in liquid nitrogen and homogenized using a Teflon pestle. After centrifugation (12,000×g), supernatant was stored at −80°C for later determinations.

2.3. Lipid plasma analysis

Concentrations of plasma TC and TG were measured using a standard colorimetric method (Sigma Chemical, St. Louis, MO, USA).

2.4. Lipid peroxidation in plasma and retinal homogenate based on measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were measured in plasma and retinal homogenates as indicators of lipid peroxidation [25]. The protein concentration was determined using a modified Bradford assay (Bio-Rad, Hercules, CA, USA) [26].

2.5. Electron microscopy

The samples from two animals in each group were processed for conventional electron microscopy; the animals were perfused for 10 min with saline plus heparin and then prefixed with 4% glutaraldehyde for 10 min. The whole enucleated eyes were fixed in 2.5% glutaraldehyde, 0.1 mol/L cacodylate and 0.2 mol/L phosphate buffered saline for 6 to 8 h. The posterior portion of the eye was dissected, washed overnight in 0.1 mol/L sodium-cacodylate buffer

(pH 7.3) containing 0.25 mol/L sucrose, postfixed in 1% osmium tetroxide, stained with 1% uranyl acetate and embedded in Epon Eraldite resin. Sections 1 μm thick were cut with an ultramicrotome, stained with toluidine blue O and examined under light microscopy to determine the areas of interest. Sections 50 to 90 nm were cut, collected on copper grids and stained with 4% uranyl acetate and lead citrate. Subsequently, the sections were examined in an EM10 Carl Zeiss transmission electron microscope (TEM) (Carl Zeiss, Thornwood, NY, USA), and images were photographed for later analysis.

2.6. Statistical analysis

All data are expressed as the mean±S.E.M. Kruskal–Wallis test was applied to assess differences among treatment groups followed by Mann–Whitney test for comparisons between two groups. $P<.05$ was considered significant. Analysis was performed using the computer program SigmaStat (v. 2.0, Jandel GmbH Erkrath, Germany).

3. Results

3.1. Murine characteristics

The apoE−/− mice were significantly heavier than the wild-type mice ($P<.05$) at the beginning and the end of the experiment. As expected, genetically modified mice (AE-C group) showed significant increases in the TC and TG concentrations ($P<.001$ and $P<.05$, respectively) compared with the WT-C group.

All mice had similar changes in weight throughout the experiment, and lutein or egg yolk supplementation did not result in a significant change in weight. Surprisingly, dietary supplementation with egg yolk, containing approximately 0.4% cholesterol, did not increase the plasma TC concentration in wild-type or apoE−/− mice, an animal model highly susceptible to dietary lipids (Table 1). Wild-type and apoE−/− mice that received egg yolk had significantly lower TG concentrations in plasma ($P<.001$ and $P<.05$, respectively), compared with control animals that received vehicle. Lutein supplementation also resulted in decreased TG values in wild-type ($P<.01$) and showed a marginal decrease in apoE−/− mice (Table 1).

Table 1
Body weight and plasma lipid profile obtained from all experimental groups at the end of the experiment

Group	Weight (g)	TC (mg/dl)	TG (mg/dl)
WT-C	28.3±3.3	69.9±9.5	83.2±3.93
WT-E	30.6±3.3	67.2±15.0	47.2±4.50*
WT-L	29.9±4.4	59.1±16.3	57.0±5.05*
AE-C	35.6±3.7*	624.7±56.3***	132.4±5.03*
AE-E	34.6±2.1	647.9±43.7	90.8±8.57†
AE-L	35.6±2.3	675.7±63.3	123.1±5.33

Data are expressed as the mean±S.E.M. Statistically significant differences from the WT-C group are indicated as *$P<.05$ or ***$P<.001$, and from AE-C group as $^{\dagger}P<.05$.

Table 2
Plasma and retinal lipid peroxidation obtained from all experimental groups at the end of the experiment

Group	Lipid peroxidation (MDA)	
	Plasma	Retina
WT-C	1.22±0.20	3.02±1.00
WT-E	1.35±0.25	4.92±3.30
WT-L	1.39±0.47	5.12±2.62
AE-C	1.66±0.32*	8.80±2.77*
AE-E	1.31±0.38†	9.10±3.04
AE-L	1.47±0.22	7.77±1.15

Data are expressed as nanomoles per milligram of protein (MDA)±S.D. Statistically significant differences from the WT-C group are indicated as *$P<.05$ and from AE-C group as $^{\dagger}P<.05$.

3.2. Effect of lutein and egg yolk supplementation on lipid peroxidation

Induction of oxidative stress by genetic hypercholesterolemia was assessed by measuring the formation of adducts with thiobarbituric acid. Plasma lipid peroxidation in the AE-C group was higher than in the WT-C group ($P<.05$) (Table 2), as expected in this model of murine hypercholesterolemia. Egg yolk supplementation decreased the lipid peroxide concentration in the plasma of apoE−/− mice down to control values ($P<.05$; Table 2). In contrast, lutein supplementation did not reduce significantly this parameter. In wild-type mice, no changes in plasma lipid peroxidation were observed in any experimental groups.

Similar to that observed in plasma, retinal lipid peroxidation in the AE-C group was much higher than in the WT-C group ($P<.01$). However, lutein and egg yolk did not modify retinal lipid peroxidation in the wild-type or apoE−/− mice.

3.3. Effect of lutein and egg yolk supplementation on retinal ultrastructural alterations in apoE−/− mice

TEM was used to determine whether the biochemical changes were associated with retinal morphologic alterations.

Before obtaining electron micrographs, we analyzed the sections stained with toluidine blue under a light microscope and observed that the AE-C group had severe vacuolization of the RPE cells compared with the WT-C group (data not shown). Subsequently, when we analyzed those changes more precisely with TEM, the eyes from AE mice had several morphologic changes mainly in the RPE and BrM; the micrographs showed disrupted cellular components and a less organized structure (Fig. 1A–F) compared with the WT-C group (Fig. 1G and H). The most important finding was the presence of basal laminar deposits (BLamD) among the basal infoldings on the RPE side of BrM, in the extracellular space between the basal lamina of the RPE, and in the inner collagenous layer of BrM (Fig. 1A and B). Microphotographs of lower magnification showed increases in the number and size of empty and autophagocytic cytoplasmic vacuoles (Fig. 1C). Several RPE cells had

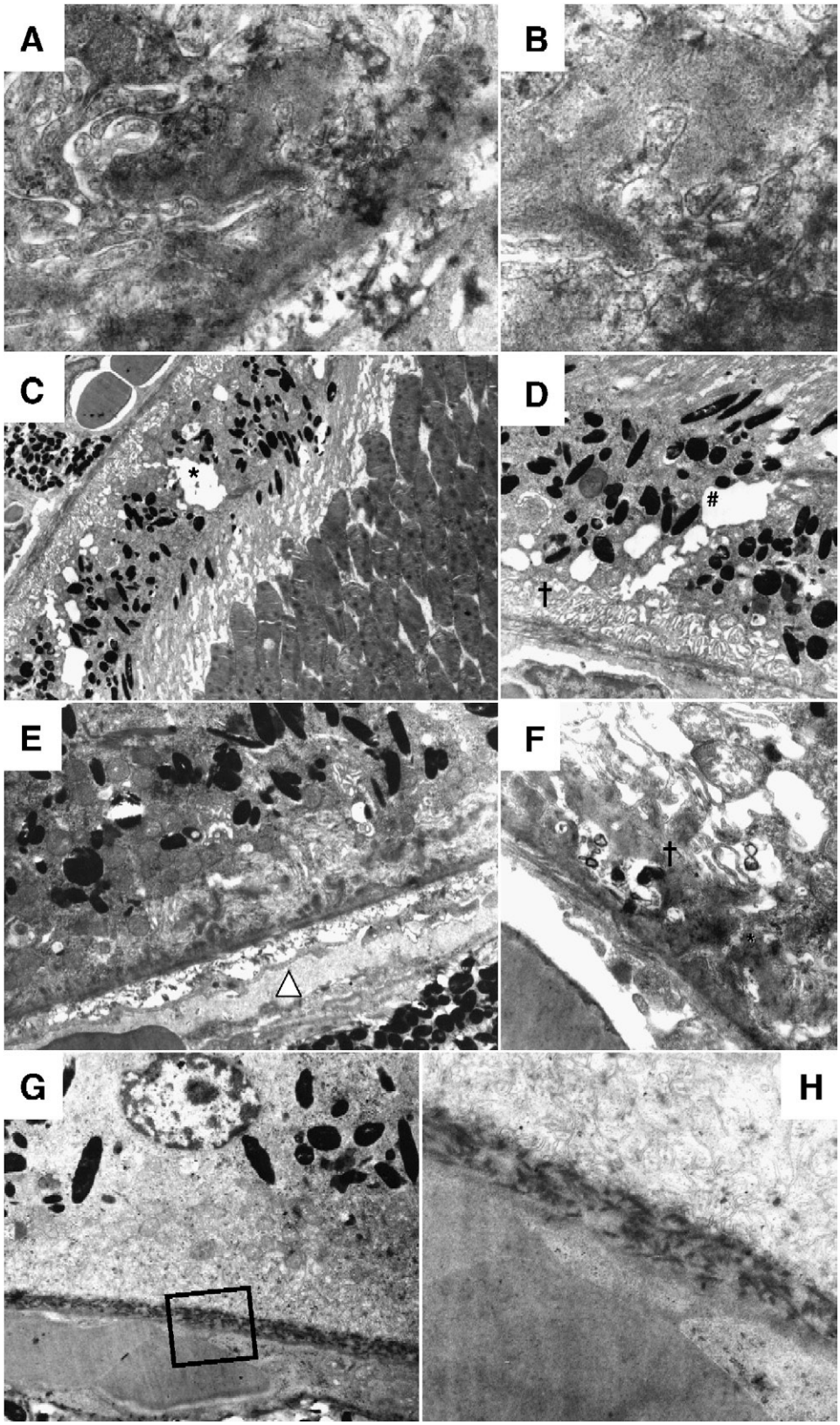

Fig. 1. Transmission electron micrograph of the outer retina and choroid of AE (A–F) and wild-type control (G and H) mice. (A, ×18 750; B, ×37 500) The AE-C group has BLamD among the basal infoldings. Separation of tight junctions between the RPE cells was patent (* in C, ×2250; # in D, ×3975) and swelling of the basal infoldings († in D) was observed. Some sections exhibited a thickened BrM in some areas (arrowhead in E, ×3975) and electrodense amorphous material among the basal infoldings († in F, ×15 000). The WT-C group has no alterations in BrM or RPE cells (G and H, ×3975 and ×15 000, respectively).

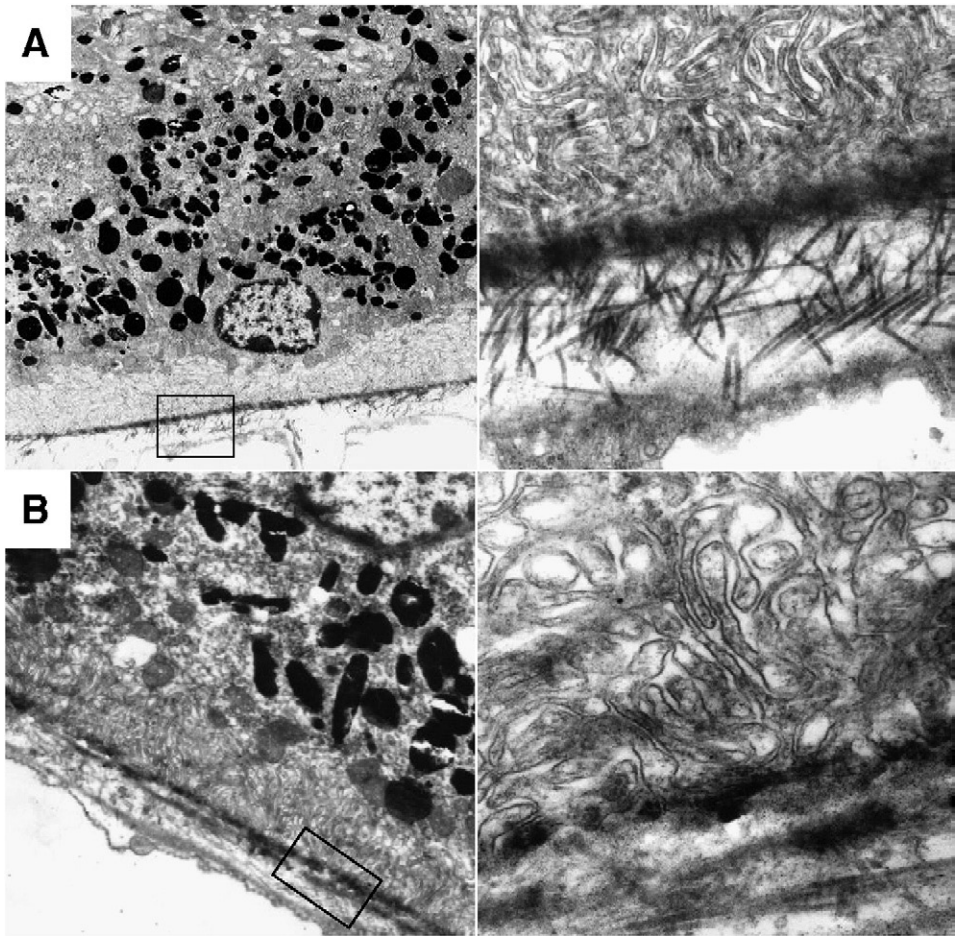

Fig. 2. Transmission electron micrographs of the outer retina and choroid of groups supplemented with lutein showed no ultrastructural alterations in either WT-L [A, ×3975 (left) and ×18 750 (right)] and less severe alterations in AE-L [B, ×3975 (left) and ×18 750 (right)] compared with the AE-C mice. Areas squared in Panels A and B are shown at higher magnification in their right-hand side.

swelling of basal infoldings and separations of the intercellular tight junctions between the RPE cells (Fig. 1D). There was also a noticeable increase in BrM thickness that was colocalized with the BLamD (Fig. 1E) and abnormal deposits of amorphous material in the sub-RPE space confined to a small area (Fig. 1F). BrM from the apoE−/− untreated animals had nonmembrane-bound vacuoles dispersed throughout both collagenous layers.

In contrast with their untreated counterparts, lutein and egg yolk groups exhibited a reduction in the severity of ultrastructural alterations in apoE−/− mice, at least in photographs analyzed. Cytoplasmic vacuolization in animals that received supplements decreased compared to the apoE−/− control group and there was no separation of the intercellular junctions in the RPE cells. Moreover, the BLamD were less intense than in controls, and BrM thickening was not observed, at least in the slices studied. Therefore, these experimental groups had RPE and BrM structures similar to the mice in the WT-C group (Fig. 2A and B; Fig. 3A and B).

No drusen or signs of neovascularization were observed in any sections analyzed.

4. Discussion

The main finding in this study was that egg yolk supplementation reduced TG and systemic lipid peroxidation in a murine model of hypercholesterolemia, surprisingly with no increases in TC. An overall improvement in retinal ultrastructural alterations was appreciated in apoE−/− mice that received lutein or egg yolk, even though there were no significant effects on retinal lipid peroxidation.

ApoE−/− mice exhibited a marked increase in TC and TG concentrations in plasma compared with their wild-type counterparts, as expected in this type of mouse [8,11,27]. In addition to the lipid profile alterations, these animals also had increased systemic oxidative stress compared with wild-type mice, assessed as TBARS in plasma, and accompanied by an increase in retinal lipid peroxidation not previously reported in this murine model. Increased retinal TBARS also had been described in experimental models of retinopathy, such as the diabetic rat, and we recently reported a similar observation in a porcine model of dietetic hypercholesterolemia [28]. Moreover, our observation agrees with evidence suggesting that abnormal lipid levels

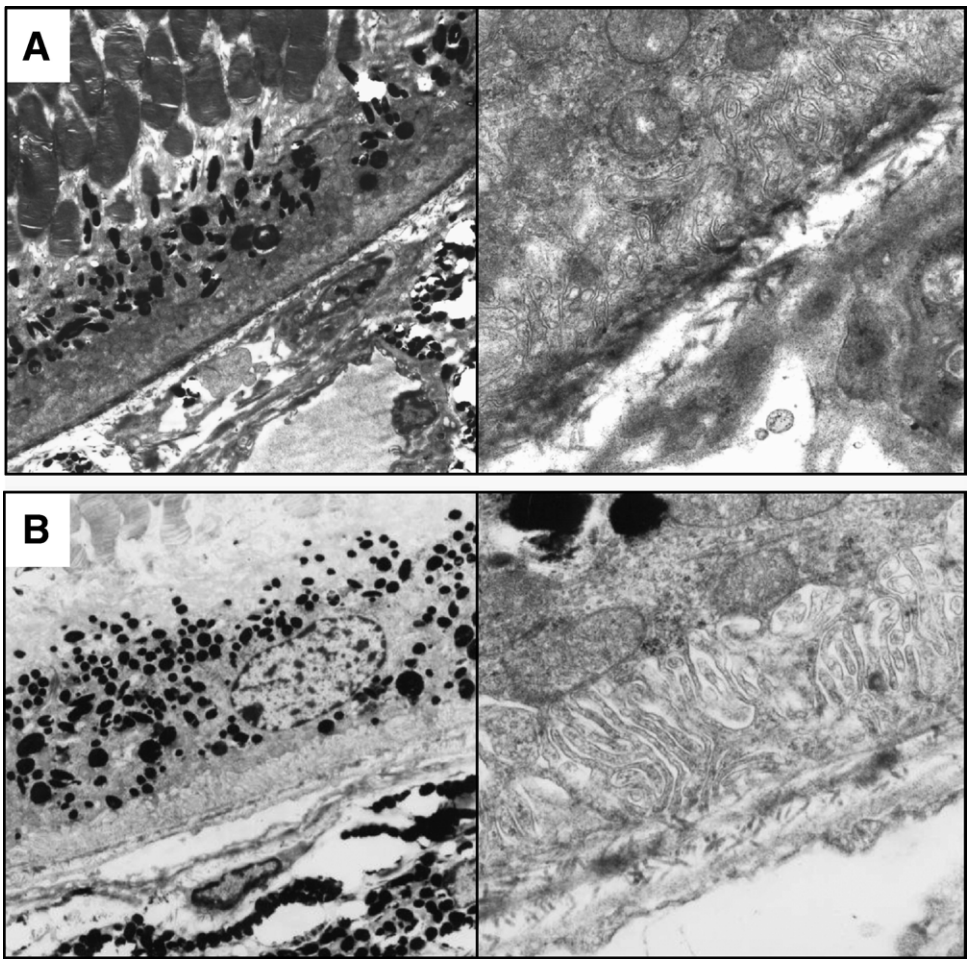

Fig. 3. No ultrastructural alterations were observed in the outer retina and choroid in wild type animals supplemented with egg yolk, and less severe alterations were observed in the AE-E group compared with AE-C animals. Panel A, WT-E [×3975 (left) and ×15 000 (right)]; Panel B, AE-E [×3975 (left) and ×15 000 (right)].

may contribute to the development of ARMD either directly or by promoting vascular disease [4,29]. Oxidative stress has been associated with a variety of degenerative ocular pathologies, such as ARMD, and the apoE−/− mouse model has been proposed as useful to study this pathology because of the retinal alterations [4].

We performed electron microscopy to assess whether biochemical changes in apoE−/− mice were linked to ultrastructural retinal alterations. We confirmed that apoE−/− mice develop several ultrastructural alterations in the retina and RPE. A major finding was BLamD formation in the RPE side of BrM, also previously observed in other murine models [6,8,30] and in a porcine model of hypercholesterolemia [28], which share similarities with altered RPE basement membranes associated with choroidal neovascularization in human ARMD. Hypercholesterolemia may contribute significantly to the retinal abnormalities, since extracellular lipid metabolism is the source of cholesterol for photoreceptors and RPE. Abnormal lipoprotein metabolism, stemming from high plasma cholesterol and a deficiency of apoE, could lead to a disturbance in the cholesterol balance in the retinal cellular layers and explain the changes in BrM in the apoE−/− mice. Lipid peroxidation may be one of the potential pathophysiologic mechanisms involved, such that BLamD formation could reflect a final common pathway of reparative responses shared by many cell types in response to injury [30]. Moreover, Kliffen et al. [24] found BLamD-like material in apoE3 transgenic mice, suggesting a pathophysiologic link between abnormal lipid metabolism and subretinal deposits.

Among the observed alterations, BrM thickening is the first and one of the major aging-associated features in deteriorating retinas. Observed thickening of the RPE basement membrane also has been reported in most proposed rodent models of ARMD [31]. Furthermore, vacuoles observed along the tight junctions between adjacent cells, together with the separation of the RPE intercellular junctions, could indicate loss of barrier function. These features, which are absent in wild-type mice and thus attributable to hypercholesterolemia, also have been reported in aging rodent and human RPE [9].

Based on biochemical and structural analyses, the apoE−/− murine model could be a valuable tool for investigating several preventive therapies. We explored the

potentially beneficial effects of lutein and egg yolk in our murine model to understand the contribution of oxidative stress to retinal pathologies, such as ARMD. Because they are pigmented substances, visible light absorption by lutein and zeaxanthin constitutes one of the mechanisms of action proposed for the preventive and antioxidant capacity of carotenoids [32]. The dose of lutein currently used in clinical setting provided by supplementation is 6 mg/day. This dose is equivalent to the 0.09 mg/kg per day that we have used in the lutein-supplemented animals in order to reproduce the dose used in the prophylaxis of ARMD. Since one egg yolk contains approximately 330 μg of lutein and zeaxanthin, 20 egg yolks would be necessary to raise 6 mg of lutein! However, the amount of egg yolk that is usually included in a regular diet is not more than two egg yolks per day. The dose of egg yolk provided to the egg-supplemented animals (0.8 g/kg per day) is equivalent to two egg yolks per day in an adult human. The lutein present in the egg yolk suministered is 0.013 mg/kg per day. Moreover, recently, Chung et al. [33] observed higher serum lutein response after subjects consumed spinach or lutein-enhanced eggs compared with when they consumed an equal dose of lutein from supplements. These findings suggest that lutein may be more bioavailable from a food matrix than from supplements [33]. However, we did not find a significant effect of lutein on the murine plasma lipid profile, in contrast with the reduction in TG observed in apoE−/− and wild-type mice that received egg yolk. Moreover, plasma cholesterol did not increase in apoE−/− mice that received egg yolk, despite the introduction of an additional source of cholesterol. This unexpected result might be explained through the inhibitory effect of egg phosphatidylcholine and sphingomyelin on the intestinal absorption of cholesterol and other lipids, reported in a rat model, and suggests that egg yolk nutrients could reduce intestinal absorption of cholesterol [34,35].

Contradictory results have been reported on the effect of lutein on systemic lipid peroxidation. Urine malondialdehyde (MDA) excretion was not significantly modified in Wistar rats supplemented with lettuce (containing 176 μg of lutein per gram of dry weight) [36], and oxidative stress biomarkers did not decrease in healthy human adults after carotenoid supplementation [37]. However, lutein supplementation reduced plasma lipid hydroperoxide [13(s)HPODE] levels in apoE−/− mice by 30% [38]. Moreover, in humans, lutein supplementation has been associated with a systemic decrease in DNA oxidative damage (8-OHdG), increased macular pigment optical density [39] and overall improvement in visual function [40].

The absence of noticeable effects of lutein on systemic lipid peroxidation is in contrast to the reduction in plasma lipid peroxidation found in apoE−/− mice that received egg yolk. However, egg yolk consumption by humans does not alter the low-density lipoprotein (LDL)/high-density lipoprotein ratio or the susceptibility of LDL to oxidation; however, the LDL size changes to a less atherogenic particle [41]. Hypothetically, the reduction in systemic oxidative stress caused by egg yolk in our model could be attributed not only to the improvement in the lipid profile but also to the effect of the antioxidant molecules present in the egg yolk (tocopherol, lutein and zeaxanthin).

The fact that egg yolk supplementation reduced systemic lipid peroxidation to control levels while retinal TBARS remained similar to that in the AE-C group could partly reflect ocular exposure to a higher risk of oxidative stress, as the result of the continuous exposure of the eye to light and, therefore, potential photooxidation. Although we did not identify studies addressing the effect of lutein or egg yolk on retinal lipid peroxidation, other authors have reported that xanthophylls protect liposomes, adult RPE (ARPE-19) cells and human lens epithelial cells against oxidative stress [42]. Although the use of CO_2 to euthanize the mice may have affected the redox state [43], the rapid depressant, analgesic and anesthetic effects of CO_2 are well established and it is an acceptable method for euthanasia of small laboratory animals.

Lutein and egg yolk supplementation resulted in a reduction in the severity of ultrastructural alterations in apoE−/− mice. Cytoplasmic vacuolization in animals that received supplements decreased compared to the apoE−/− control group and there was no separation of the intercellular junctions in the RPE cells. Moreover, the BLamD were less intense than in controls, and BrM thickening was not observed, at least in the slices studied. The divergent lipid and redox responses to egg and lutein treatment, yet similar effects on the retinal histopathology, suggest that the beneficial effects in the retina may be due to the light-absorbing properties of lutein and possibly zeaxanthin, rather than effects on lipid metabolism or redox state [32]. Isolated supplementation of lutein or egg yolk is not efficient in complete protection of ocular ultrastructure in cases of oxidative stress related to hypercholesterolemia. Because of the limited number of animals used for histological examination, a subtle difference between egg yolk and lutein effects on retinal ultrastructure may have been underestimated. Nevertheless, the design of this study was mainly focused on biochemical parameters. Our results confirm previous reports on the beneficial effect of vitamin E in a more extreme model, the light-damaged mouse with combined genetic and long-term dietetic hypercholesterolemia [44], and of supplementation with vitamins C and E in a porcine model of hypercholesterolemia [28].

All of these results suggest that egg yolk has potential antioxidant properties, probably because of lutein, zeaxanthin and other compounds in its lipid matrix that protect the retina against oxidative damage. It has been reported that zeaxanthin is more active than other carotenoids in preventing lipid oxidation, and the authors hypothesized that the direct reaction of peroxynitrite with zeaxanthin and other carotenoids is less important in vivo than reactions of peroxynitrite with other targets in the lipid bilayer [45]. In some situations, this antioxidant effect of single carotenoids

may be synergistic with the antioxidant effects of other carotenoids and other compounds [46]. However, definitive conclusions require assessment of other oxidative stress biomarkers, such as 8-OHdG, nitrotyrosine and isoprostanes, to arrive at a conclusion about the protective effects of lutein and zeaxanthin against degenerative retinal processes.

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