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Research article

# Age-related changes of the human retinal vessels: Possible involvement of lipid peroxidation

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ABSTRACT

*Background:* Aging of the human retina is accompanied by oxidative stress that exerts profound changes in the retinal neurons. It is unknown if oxidative stress influences the cellular components of the retinal vessels in some ways.
*Methods:* We examined changes in retinal vessels in human donor eyes (age: 35–94 years; $N = 18$) by light and transmission electron microscopy, TUNEL and immunohistochemistry for biomarkers of vascular smooth muscle cells (SMC; actin), oxidative stress (4-hydroxy 2-nonenal [HNE] and nitrotyrosine), microglia (Iba-1) and vessels (isolectin $B_4$).
*Results:* The earliest changes in the endothelium and pericytes of capillaries are apparent from the seventh decade. With aging, there is clear loss of organelles and cytoplasmic filaments, and a progressive thickening of the endothelial and pericyte basal lamina. Loss of filaments, accumulation of lipofuscin and autophagic vacuoles are significant events in aging pericytes and SMC. Actin immunolabelling reveals discontinuity in arterial SMC layers during eighth decade, indicating partial degeneration of SMC. This is followed by hyalinization, with degeneration of the endothelium and SMC in arteries and arterioles of the nerve fibre layer (NFL) and ganglion cell layer in ninth decade. Iba-1 positive microglia were in close contact with the damaged vessels in inner retina, and their cytoplasm was rich in lysosomes. HNE immunoreactivity, but not of nitrotyrosine, was detected in aged vessels from seventh decade onwards, suggesting that lipid peroxidation is a major problem of aged vessels. However, TUNEL positivity seen during this period was limited to few arteries and venules of NFL.
*Conclusion:* This study shows prominent age-related alterations of the pericytes and SMC of retinal vessels. These changes may limit the energy supply to the neurons and be responsible for age-related loss of neurons of the inner retina.



## 1. Introduction

Aging is associated with various degenerative changes in the retina. These include early morphological and ultrastructural changes of neurons to gradual loss of altered neurons (Gao and Hollyfield, 1992; Curcio, 2001; Nag et al., 2006; Aggarwal et al., 2007; Pow and Sullivan, 2007; Nag and Wadhwa, 2012, 2016). Aging is responsible for the development of certain retinal diseases, such as age-related macular degeneration (AMD) and diabetic retinopathy (DR), wherein the vasculature components undergo remarkable changes. Clinically, vascular complications with aging are believed to be responsible for causing visual problems in the aged population (Pizzarello, 1987; Minaker, 1987).

Not only are there changes in the sensory retina with aging, but also the supporting glial cells (e.g., Müller cells) show certain changes. Müller cells that play a pivotal role in supplying nutrients and removal of wastes of retinal neurons, undergo oxidative damage with aging in humans (Nag et al., 2011) and experimental animals (Curtis et al., 2011). Because they are intimately associated with retinal vessels, it is probable that vascular elements are also prone to be damaged by oxidative stress (OS). Importantly, one study reported that Müller cells trigger capillary changes in aging human retina (Bianchi et al., 2016). It is currently unclear how OS influences the retinal vasculature and causes alterations. Since OS is involved in triggering complications in diabetes (Baynes, 1991; Curtis et al., 2011) and many features of retinal vessels in aged rats mimic with those seen in experimental rats with diabetic retinopathy (Roy et al., 2010, 2017), therefore, in this study, we

*Abbreviations:* BL, basal lamina; GCL, ganglion cell layer; HNE, 4-hydroxy 2-nonenal; INL, inner nuclear layer; SMC, smooth muscle cells.

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have examined various age-related changes of the human retinal vessels and whether they show any link with OS.

## 2. Materials and methods

### 2.1. Tissues

Human eyes from eighteen donors (Table 1) were procured from the National Eye Bank, Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi via approval of the institutional Human ethics committee (IHEC No. IEC/NP-57/2010). Informed consent was obtained from the relatives of the deceased for permission and use of the eyes in research. Based on the availability of eyes from the donors, those below 72 years of age served as relatively lower age group (age range: 35–72 years; group A), compared to those from the advanced aged group (age range: >72 years; group B). The eyes were fixed and the retinas processed for light and transmission electron microscopy (TEM), TUNEL and immunohistochemistry (IHC) to examine vascular changes, dying vessels and expression of certain biomarkers of OS in the vessels in different age groups.

### 2.2. Light microscopy and TEM

The left eyes were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 4 h at 4 °C. After wash, the macular, temporal peripheral part (eccentricity: 3–6 mm from the macular border) and nasal part of the retina (3–4 mm away from the optic disc) were chopped in a temporal–nasal direction via the optic disc. The retinal samples were postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, infiltrated and embedded in Araldite CY 212. Thick sections (0.5 μm) were stained with toluidine blue and examined under a light microscope to find gross changes in the vessels. Thin sections (60–70 nm) from those samples were cut, stained with aqueous uranyl acetate and lead citrate and examined under a Tecnai G2 20 S-Twin transmission electron microscope (FEI Company, Eindoven, The Netherlands).

### 2.3. Morphometry

The changes in the thickness of the basal lamina (BL) of 10 capillaries located in the ganglion cell layer (GCL) and/or inner nuclear layer (INL) of macula were assessed. Measurements were made in five randomly collected sections retrieved from different depth of the blocks, each section was separated by a distance of 10 μm trimmed from the block face after a section was retrieved. The vessels in the nerve fibre layer (NFL) were not considered due to frequent tears that developed during imaging of this layer. The images were acquired at a magnification of 4500–7000× using DigitalMicrograph software equipped with the microscope. From each retina, the measurements of endothelial and pericyte BL were performed at two points of capillaries cut in spherical profiles and the mean value ± standard deviation (SD) of the parameters was calculated for each retina. Abnormally thick BL with accumulation of debris and resultant splitting was not considered in such measurements. The data were pooled together into a relatively lower age group (donor ages: 35–72 years) and higher age group (>72 years) and compared.

### 2.4. TUNEL

Apoptosis was detected by TUNEL using In situ cell death detection kit (Roche Applied Science, Mannheim, Germany). Frozen retinal sections were washed, permeabilized in 0.1% Triton X-100 and incubated in TUNEL reaction mixture for 60 min at 37 °C in a dark humidified chamber. Sections were developed by treating in 0.06% diaminobenzidine tetrahydrochloride hydrate (DAB) and 0.05% hydrogen peroxide. Negative controls were incubated with the label solution only, while positive control sections were initially treated with 1% deoxyribonuclease (DNAse) and then in the TINEL reaction mixture. The sections were dehydrated, mounted and visualized under an optical microscope (Leica DM6000 B). Brown signals in nuclei of vascular elements were considered to be TUNEL positive.

### 2.5. Immunohistochemistry

The right eyes were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 12 h at 4 °C. After wash, the retina was cut in a nasal–temporal axis via the optic disc and macula. The retina was cut for a length of 3–5 mm nasal from the optic disc and 10 mm along the temporal axis, which included the macula and peripheral part (eccentricity: 3–7 mm from the macular border). The samples were cryoprotected in 15–30% sucrose and frozen sections (thickness: 14 μm) cut. They were mounted serially onto gelatin-coated slides and stored at −20 °C.

For immunolabelling, sections were treated in 0.3% hydrogen peroxide in methanol for 30 min to quench the endogenous peroxidase activity. These were incubated in 10% normal serum (diluent: 0.01 M phosphate-buffered saline containing 0.5% triton X-100) for 2 h and then in primary antibodies against alpha smooth muscle actin (mouse monoclonal; Dako, Glostrup, Denmark; catalogue number: M0851, dilution: 2 μg/ml), Iba 1 (rabbit polyclonal; Abcam Plc, UK; catalogue number: ab5076; dilution: 1 μg/ml), 4-HNE (rabbit polyclonal; Alpha Diagnostic International, San Antonio, Texas, USA; catalogue number: HNE 11-S; dilution: 2 μg/ml) and nitrotyrosine (mouse monoclonal; Millipore, Billerica, USA; catalogue number: 05-233, Dilution: 5 μg/ml) for 48 h at 4 °C. Most antibodies were classified as standardized under antibody database published (The *Journal of Comparative Neurology* antibody database V.14) and also used earlier by us showing antibody specificity (Nag et al., 2017, HNE; Nag et al., 2019, nitrotyrosine). After wash, sections were incubated in the biotinylated secondary antibodies (dilution: 1:200; Vector Laboratories, Burlingame, CA, USA) for 6 h at 4 °C. Immunoreactions in sections were visualized by using the avidin–biotin immunoperoxidase method (Vectastain Elite Kit, Vector Laboratories, CA, USA) using 0.06% DAB as a chromogen. In control experiments, incubation of sections in the primary antibody was substituted with the secondary antibody. Few sections were also labelled with biotinylated isolectin B4 (Sigma-Aldrich Corporation, MO, USA, catalogue number: L2140, dilution: 1 μg/ml), to visualize the distribution and overall features of the vessels. These were developed by the standard avidin-biotin immunoperoxidase method, as outlined above. The slides were dehydrated in ethanol and coverslipped with DPX. Photographs of sections (including those obtained from resin blocks) were taken under an optical microscope (Leica DM 6000B), using software [(Leica Application Suite, Version 3.4.1; Leica Microsystem (Switzerland) Limited)].

### 2.6. Quantification

For quantification, five randomly collected sections (every tenth section) from each sample were chosen and immunolabelling performed therein. Images were acquired under an optical microscope at 2.5× magnification of the objective lens and the number of HNE positive blood vessels (located in NFL, GCL and INL) was counted per 500 μm length of the retina on digitized images. The data were pooled together into a relatively lower age group (donor

**Table 1**
Basic information on human donor eyes (18) used in this study.

| Decade | Age | Sex | Cause of death[a] | Delay in fixation |
|---|---|---|---|---|
| 4th decade | 35 | M | Road-traffic accident | 2 |
| 5th decade | 45 | M | Cardiac arrest | 3 |
| 6th decade | 54 | M | Heart attack | 2 |
| | 56 | F | Cardiac arrest | 2 |
| 7th decade | 62 | M | Cardiac arrest | 2 |
| | 63 | M | Retro-pharyngeal abscess | 3 |
| | 67 | F | Cardiac arrest | 4 |
| | 70 | M | Myocardial infarction | 2 |
| 8th decade | 72 | M | Heart attack | 2 |
| | 74 | M | Heart attack | 2 |
| | 78 | M | Myocardial infarction | 3 |
| | 79 | M | Cardiac arrest | 2 |
| 9th decade | 81 | F | Cardiac arrest | 4 |
| | 84 | M | Heart attack | 2 |
| | 88 | M | Heart attack | 4 |
| | 89 | F | Cardiac arrest | 3 |
| | 90 | F | Heart attack | 3 |
| 10th decade | 94 | F | Cardio-respiratory attack | 1 |

M, male; F, female.
[a] Retrieved from case registry.

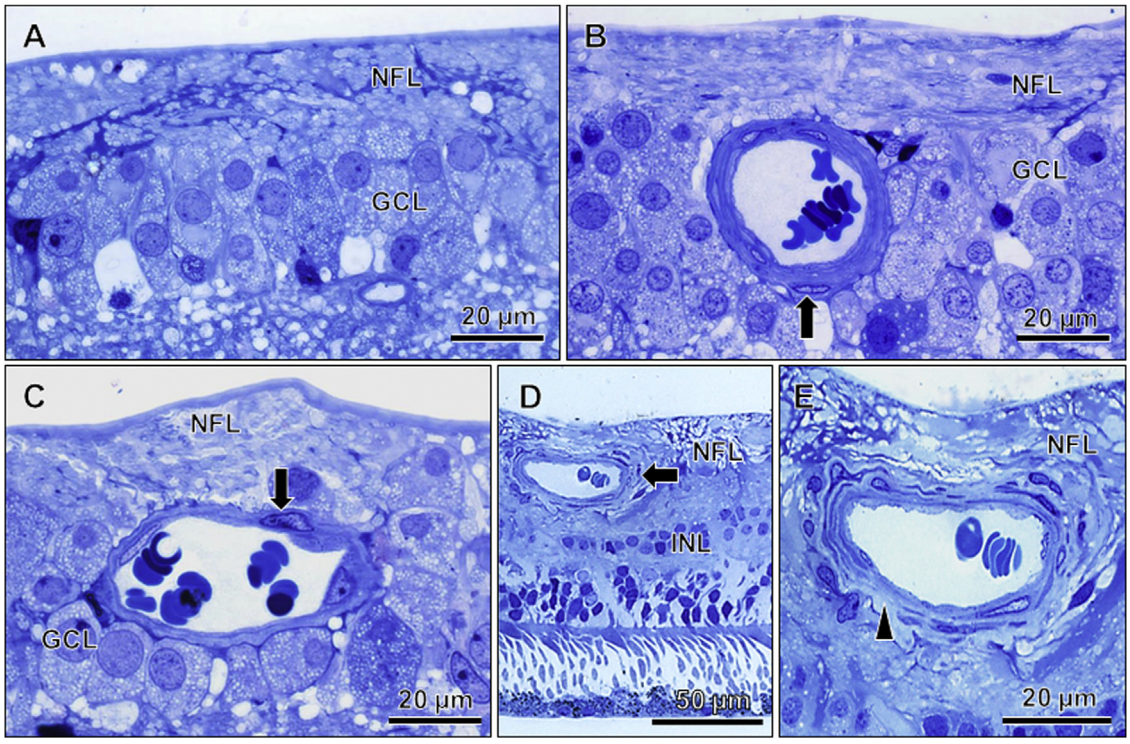


**Fig. 1.** Light micrographs showing parts of the retina and vessels and their status with aging. (A) NFL and GCL of the parafovea. An artery (B) and an arteriole (C) located in parafoveal GCL. In both, the medial SMC are intact (arrows). (D) An artery of the peripheral retinal NFL. There is a mild loss of endothelium and appearance of thickened adventitia (arrow), as shown in the enlarged view of this vessel in (E). Also note the partial disorganization of medial SMC (E, arrowhead). From a 74-year-old donor.

ages: 35–72 years) and higher age group (>72 years) and compared.

### 2.7. Statistical analysis

All data are shown as mean ± standard deviation (SD). The significance of changes of capillary BL between lower age (<72 years) and higher age group (>72 years) in donor retinas was analyzed by Student's *t*-test. The same was also used to find the significance of changes in the mean number of HNE positive blood vessels between two groups. Changes between endothelial and pericyte BL were compared and analysed by two-way ANOVA, followed by comparisons by Post hoc Tucky's analysis.

## 3. Results

### 3.1. Light microscopy

Examinations of the retinal sections under light microscope revealed early, insignificant autolytic changes (unavoidable) in the parenchymal tissue. In three donor retinas (age: 35, 45 and 56 years), the retinal arteries and capillaries appeared normal in appearance with healthy endothelium and intramural pericytes. The arteries of the NFL and GCL possessed 5–6 layers, while the arterioles had 2–3 layers of smooth muscle cells (SMC) in their medial layer. No significant changes were traced in the retinal vessels at sixth decade of life (51–60 years). Fig. 1A shows parts of the NFL

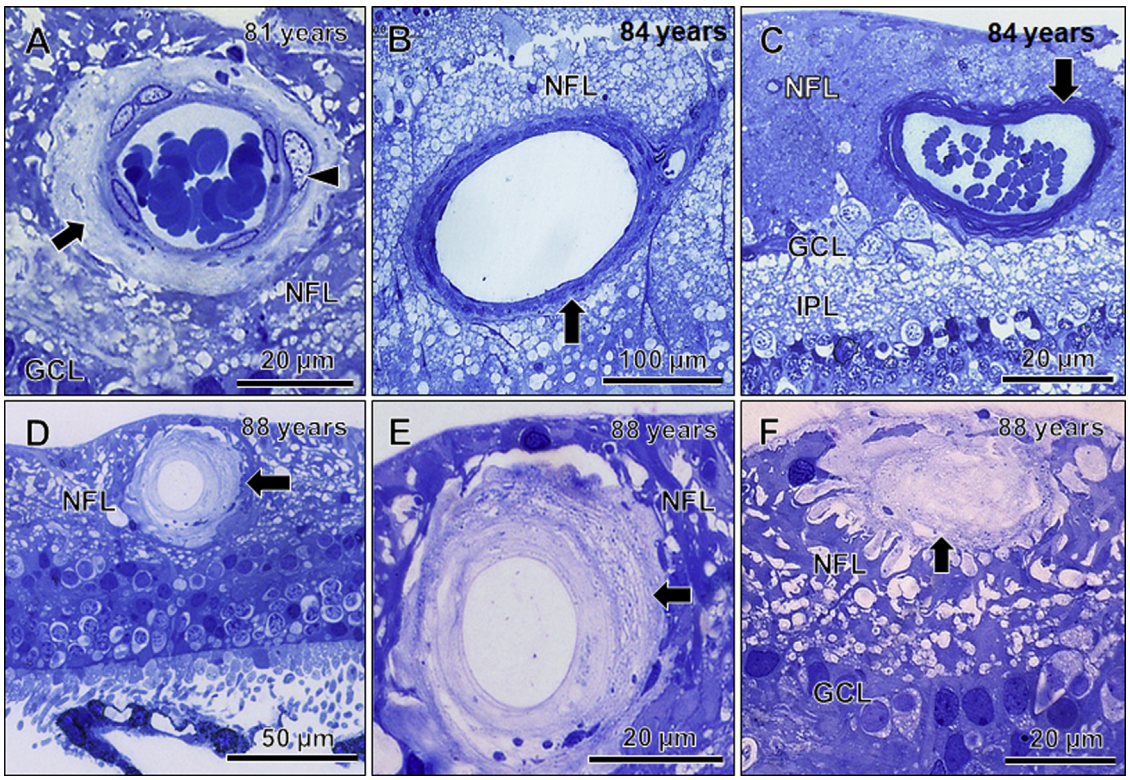


**Fig. 2.** Light micrographs showing changes of retinal vessels in advanced aging. There is a marked loss of endothelium and appearance of thickened adventitia (arrows) in arteries of NFL. While SMC are still present in (A) (arrowhead), there is complete loss of these cells with progressive hyalinization of the arterial walls (B–F). (E) is the magnified view of the vessel in (D) (arrow). The vessels are also clearly acellular. Donor ages are indicated on the top right-hand corner of the images.

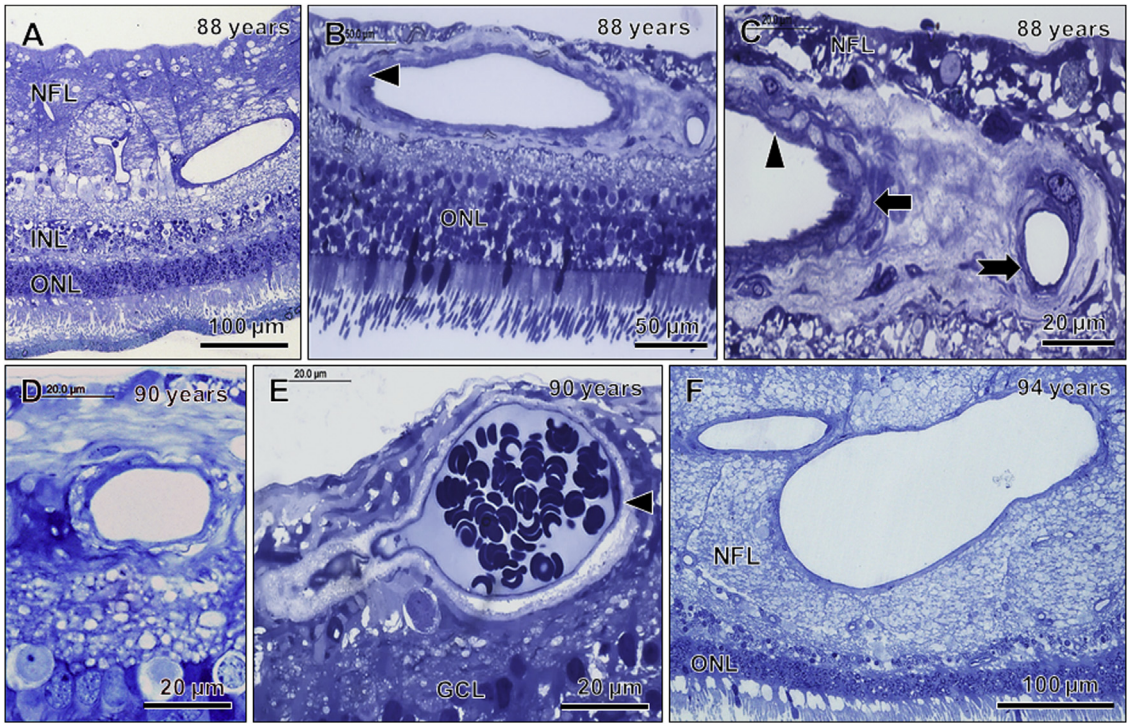


**Fig. 3.** Light micrographs showing changes of retinal arterioles (A–D), and venule (E) and vein (F) with aging. The vessels, which are located in the NFL appear irregular in outline and without endothelium in (A), (D) and (F), and there is some endothelial remnant in (B), (C) and (E) (arrowheads). There is a marked loss of SMC in (A), (D) and (E). Part of the arteriole in (B) is enlarged to show its medial wall in (C), which shows hypertrophied SMC (arrow). Its wall shows extreme fibrosis and contains a small vessel (vasa vesorum, notched arrow). INL, ONL, inner- and outer nuclear layers, respectively. Donor ages are indicated on the top right-hand corner of the images.

**Table 2**
Summary of various features and their changes seen in human retinal vessels of NFL, GCL and INL with aging (based on LM, TUNEL, IHC and TEM observations).

| Features | Age range (years) | Status/changes |
|---|---|---|
| Endothelium | 35–64 | Normal with organelles |
| | 67–74 | Partly degenerative, with vacuoles containing thin filaments |
| | 78–94 | TUNEL positivity in veins, severe to complete loss in advanced ages (>85 years) |
| Pericyte | 35–64 | Normal with ample cytoplasmic intermediate filaments |
| | 67–74 | Partial loss of filaments<br>Appearance of lipofuscin granules |
| | 78–94 | Marked degeneration with loss of filaments and accumulation of more lipofuscin |
| SMC | 35–64 | Normal with thin filaments |
| | 67–74 | Partial loss of filaments<br>Accumulation of lipofuscin |
| | 78–94 | Partial loss<br>TUNEL positivity, hyalinization of arterial wall after loss of SMC, more prominent in advanced ages (>85 years) |

| Features | Age range (years) | Status/changes | |
|---|---|---|---|
| BL thickness | 35–72 | Range: 82.05–88.21 nm<br>Mean: 84.16 ± 0.70 nm (endothelial) | Range: 82.62–89.57 nm<br>Mean: 85.30 ± 0.79 nm (pericyte) |
| | >72 years | Range: 91.34–141.47 nm<br>Mean: 111.2 ± 5.3 nm (endothelial) | Range: 90.77–242.3 nm<br>Mean: 132.6 ± 16.48 nm (pericyte)<br>Thickens late in advanced ages (>85 years) |

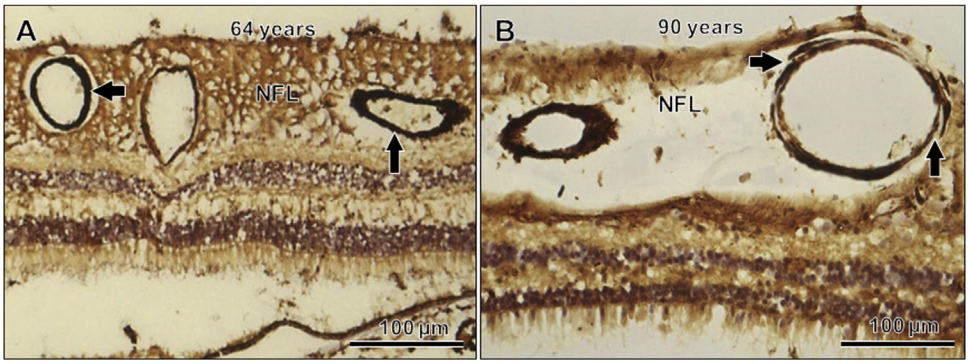


**Fig. 4.** (A, B) Actin IR in NFL vessels. The vessels in (A) appear intact in its SMC layers (arrows); they are incomplete in (B) (arrows), indicating degenerative change. Donor ages are indicated on the top right-hand corner of the images.

and GCL of the parafovea of a 75-year-old donor, wherein marked changes in large vessels were seen in advanced ages. Between seventh (61–70 years) and eighth decade (71–80 years), the arteries and arterioles located in the GCL showed mild loss of endothelium, appearance of thickened adventitia (Fig. 1B–D) and partial disorganization of medial SMC (Fig. 1E).

In arteries and capillaries, there was evidence of irregular outline and occlusion (with erythrocytes; supplementary Fig. S1). With advanced aging at the ninth (81–90 years) and tenth decade (91–94 years), there was moderate to complete loss of endothelium and SMC (Figs. 2A–F and 3A, D, E ) with marked sclerosis (Figs. 2D–F and 3C) in arteries of the NFL; the arterial SMC that survived showed hypertrophy (Fig. 3B and C). The details of the features noted in 10–12 sections in vessels from each retina are summarized in Table 2.

Actin immunoreactivity (IR) revealed intact vessels in lower ages (e.g., 64 years; Fig. 4A), but discontinuity in labelling of the arterial SMC layer (Fig. 4B), indicating degeneration of SMC. These vessels were mostly located in the NFL. Labelling with isolectin B4 showed irregular and occasional thin contours of vessels in inner retina (supplementary Fig. S2).

### 3.2. TEM

In young donor retinas (e.g., 35 and 45 years of age), the endothelium of capillaries (Fig. 5A–C) and venules (Fig. 5D) was lined by a thin BL that also surrounded the pericytes (Fig. 5A–C). Cytoplasmic organelles (viz., mitochondria, rough endoplasmic reticulum, Golgi complex and vesicles) were abundant in vascular endothelium (e.g., Fig. 5D) and pericytes. The pericytes showed an abundance of cytoplasmic filaments and dense plaques (Fig. 5C).

In the retinas of elderly individuals between the eighth and ninth decade, the capillary endothelium and pericytes were partly degenerative, with loss of organelles (Fig. 6A–C). There was an apparent loss of filaments in the pericytes (Fig. 6B), and they showed an accumulation of lipofuscin (Fig. 6C–E) and autophagic vesicles (Fig. 6F). Pyknotic nuclei (with condensed heterochromatin) of pericytes were also occasionally found (supplementary Fig. S3), indicating that pericyte may die via apoptosis. The capillary wall, being invested by Müller cell process also contained lipofuscin granules (Fig. 7A and B). While the SMC of arteries and arterioles appeared normal in lower ages (e.g., Fig. 8A), with progression of aging, they showed swelling (supplementary Fig. S4,

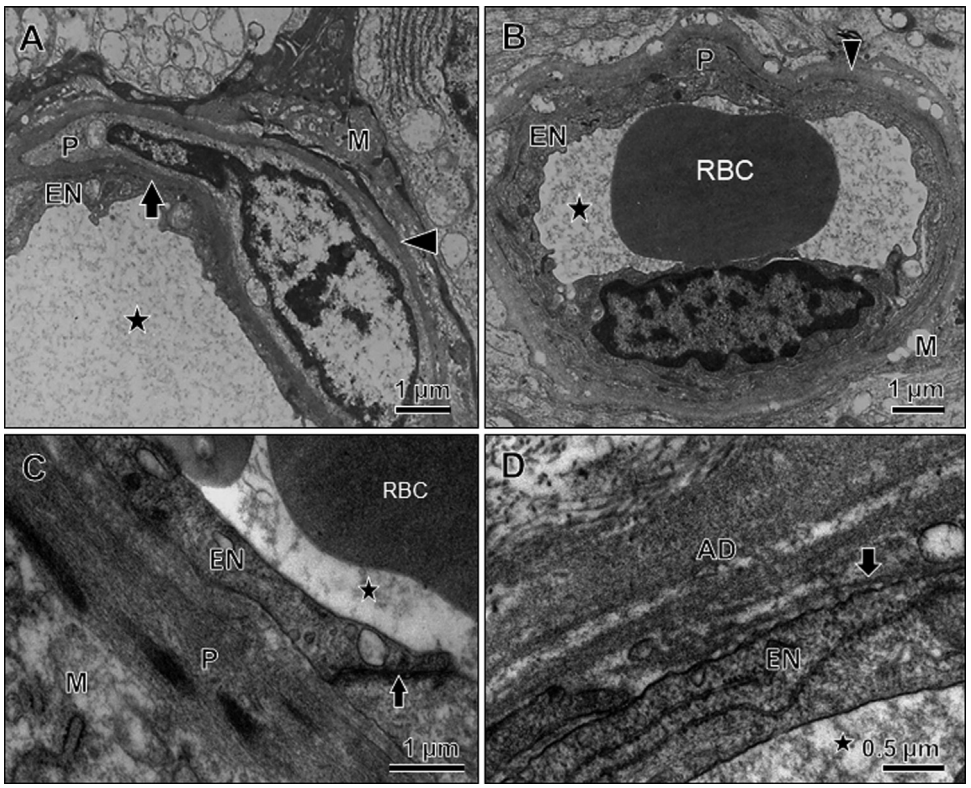


**Fig. 5.** Transmission electron micrographs of three capillaries (A–C) and a venule (D) in young donor retinas. Capillaries are lined by endothelium (EN) and pericytes (P), both of which lie on a thin BL, the arrow denotes the endothelial BL (A) and arrowhead denotes the pericyte BL (A, B). The capillary in (C) shows filaments in a pericyte (P) and dense plaques. The arrow indicates tight junction between EN. The venule (D) shows long, rough endoplasmic reticulum in EN. Its BL (arrow) merges with the adventitia (AD). Stars, lumen of vessels; M, Müller cell processes (A–C); RBC, red blood cells (B, C). From 35- (A, B) and 54-year- (C, D) old donors.

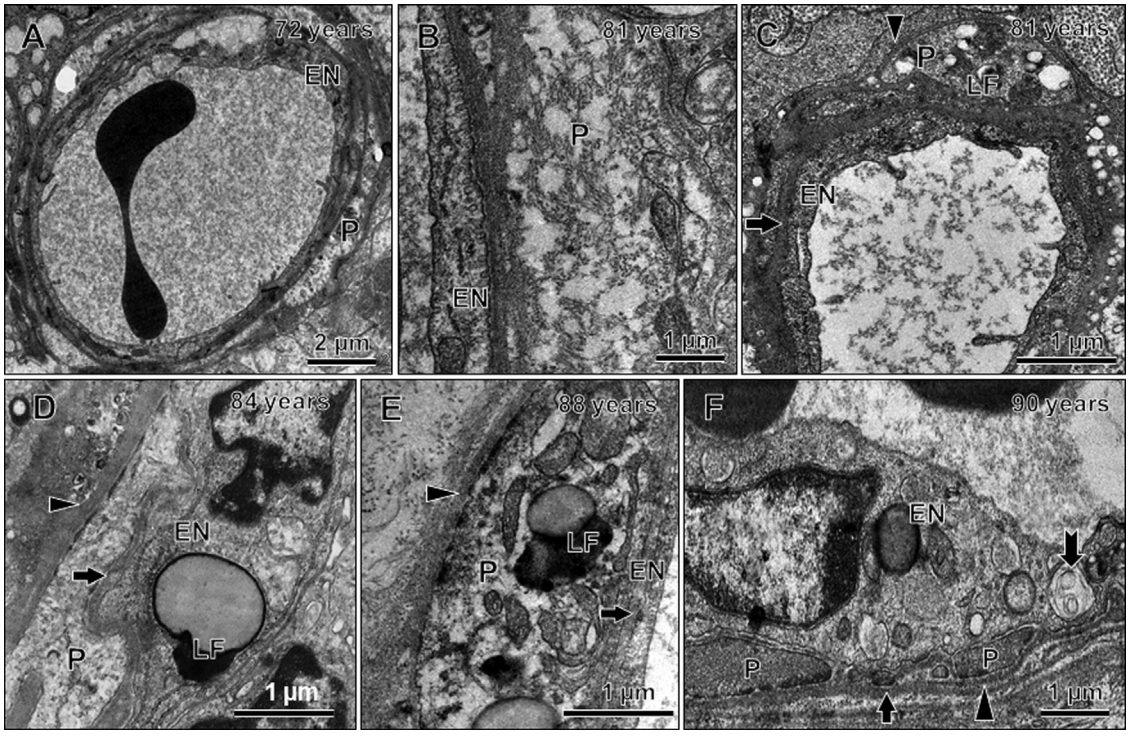


**Fig. 6.** Transmission electron micrographs showing degeneration of capillary endothelium (EN) and pericytes (P) between eighth and ninth decade. Loss of organelles is evident in EN and P (A–C), P show loss of filaments (B) and an accumulation of lipofuscin (LF, C–E) and autophagic vesicles (F, notched arrow). Arrows and arrowheads show the BL of EN and P, respectively. Donor ages are indicated on the top right-hand corner of the images.

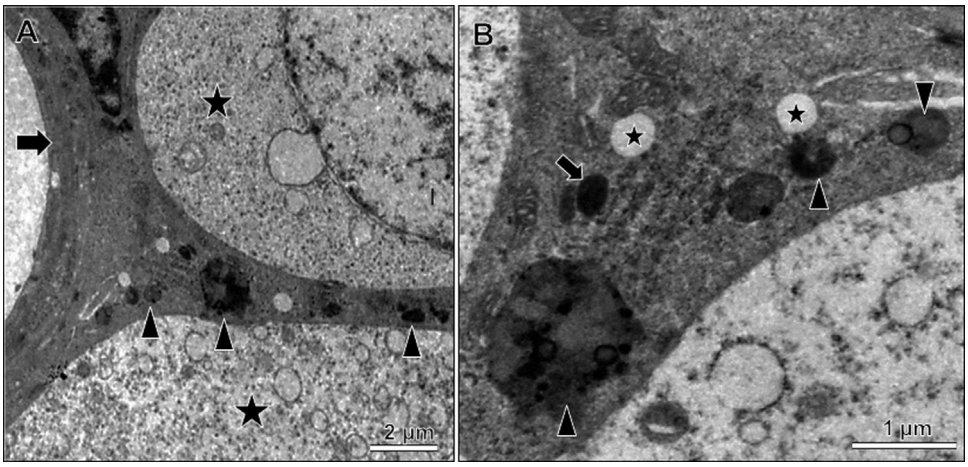


**Fig. 7.** (A) Part of a capillary (arrow), whose wall is invested by Müller cell process containing dark inclusions (arrowheads). Two degenerated INL neurons (stars) are indicated. (B) Magnified view of Müller cell process showing inclusions as lipofuscin granules (arrowheads), lipid droplets (stars) and lysosomes (arrow). From an 88-year-old donor retina.

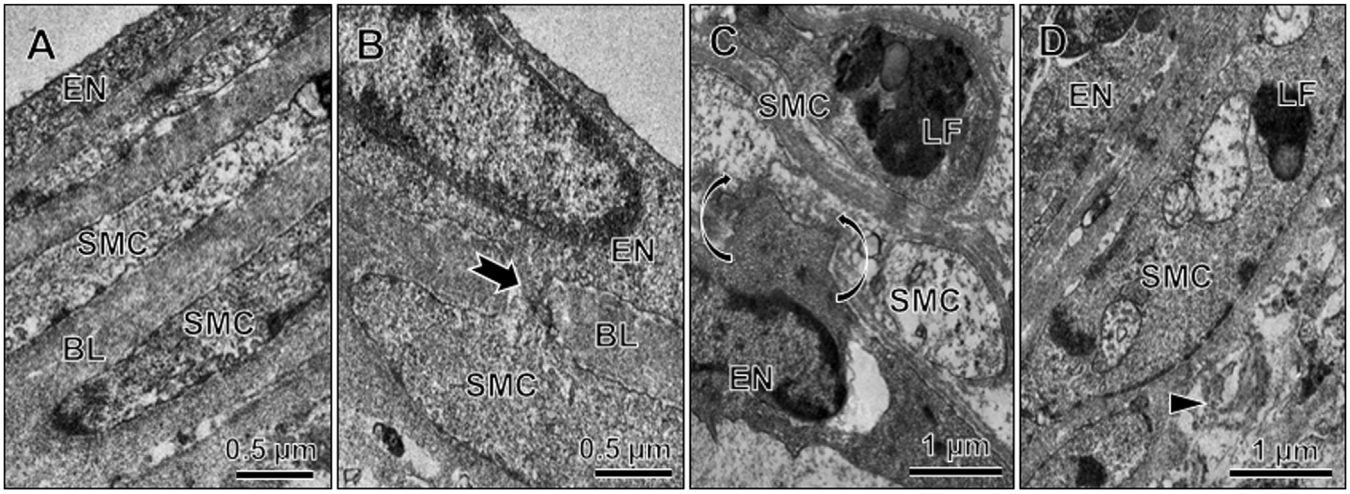


**Fig. 8.** Details of arteries and arterioles of GCL with aging. (A) Normal appearance of endothelium (EN) and SMC. (B) The BL of EN is ruptured (arrow), causing herniation of EN cytoplasm into SMC. (C, D) Partial degeneration and lipofuscin (LF) accumulation in SMC. The EN is partly degenerative; in (C), the EN projects into SMC through a break in BL (curved arrows), as in (B). From 81- (A, B), 84- (C) and 88-year (D)-old donors.

arteriole) and accumulation of lipofuscin (Fig. 8C and D, arterioles). In two cases, the endothelial BL in arterioles was found to be ruptured, allowing herniation of endothelial cytoplasm into SMC (Fig. 8B and C). Extensive degeneration of the SMC of arterioles and arteries was noted in advanced ages (Fig. 9A and B; supplementary Figs. S5 and S6). In few cases, tortuous BL of both endothelium and SMC was seen in degenerating arteries (supplementary Fig. S5). Endothelial and pericyte degeneration in venules was also seen in advanced ages (supplementary Fig. S7A and B). They showed a thicker adventitia that accumulated abnormal vesicles (supplementary Fig. S7B).

Morphometric evaluation of the thickness of the endothelial and pericytes BL (of INL capillaries) revealed that both increased significantly with aging (Fig. 10A and B). In the higher age group (>72 years), the thickness of endothelial as well as pericyte BL was significantly increased compared to that in the lower age group (Fig. 10A, $p = 0.0001$; Fig. 10B, $p = 0.01$, Student's $t$-test). When the thickness of the endothelial and pericyte BL was compared for lower age (<72 years) and higher age group (>72 years), it was found that in the lower age group, there was no significant difference in thickness between endothelial and pericyte BL; however, the latter was significantly increased in the higher age group compared to that in the lower age group (Fig. 11, $p = 0.001$, two way ANOVA, Post hoc Tucky's test).

### 3.3. Immunolocalization of markers of oxidative stress in retinal vessels

The status of HNE-IR, indicative of lipid peroxidation, was examined in aging retinal vessels. IR was absent in the retina of lower ages (e.g., 45 years; Fig. 12A), it was detected in few INL cells in the 62-year (Fig. 12B) and in vessels of the 63-year and 70-year-old donor retinas (Figs. 12C and 13A, B ). In the latter, the vessels appeared compressed (Fig. 13B). With advanced aging, IR was seen in veins (Fig. 12D), in capillaries of parafoveal GCL and IPL (Fig. 12E and F) and artery (Fig. 12G). Strong IR was also seen in Müller cell process that invested the vessel wall (Fig. 12G). Count of profiles of HNE positive vessels (cut either longitudinally or transversely) per 500 µm length of the retina revealed that the number increased in the higher age group, compared with the lower age group (<72 years; $p = 0.0014$, Student's $t$-test; Fig. 13C).

TUNEL, which indicates cellular apoptosis, showed labelling in nuclei of the arterial SMC (Fig. 14A and B) and endothelium of vein (Fig. 14B) in aged vessels. Labelling was present in intact medial

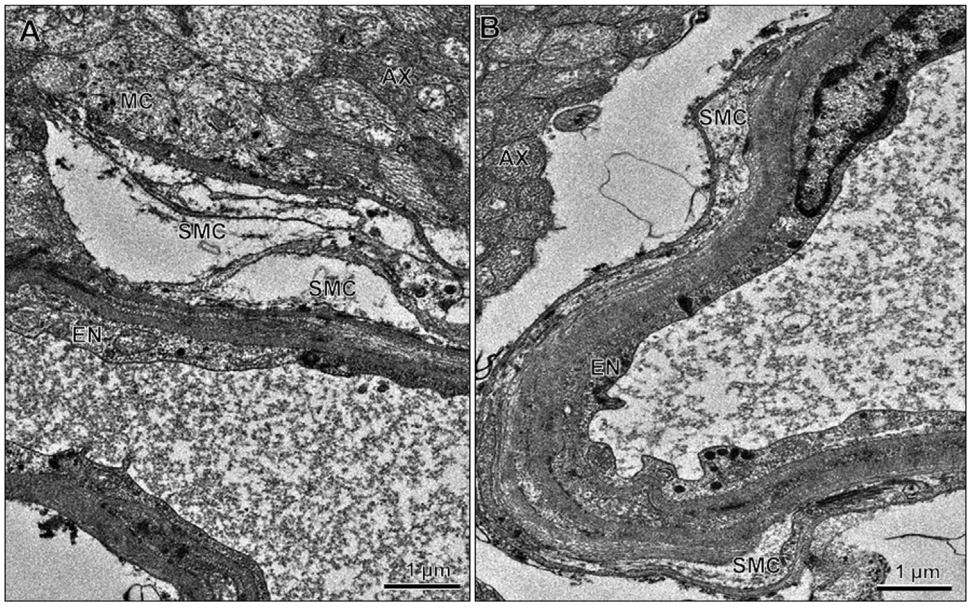


**Fig. 9.** Transmission electron micrographs of arterioles, showing SMC degeneration. The cytoplasm of endothelial cells (EN) appears normal. The adjacent Müller cell process (MC; A) and axons (AX; A, B) show normal complement of filaments, indicating filament degeneration in SMC is not artefactual. From an 89-year-old donor.

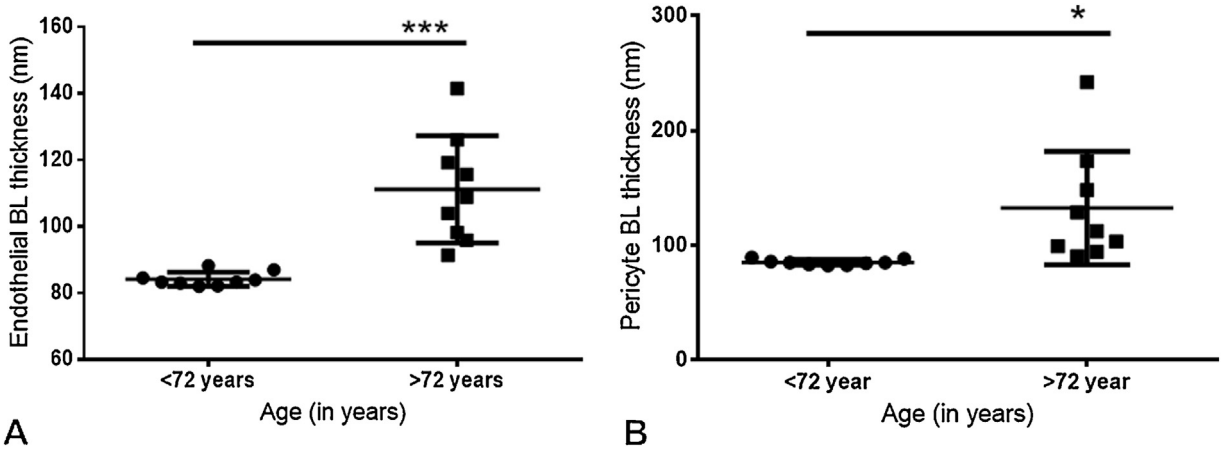


**Fig. 10.** Thickness (mean ± SD) of endothelial BL (A) and pericyte BL (B) in retinal capillaries in INL for lower age (<72 years) and higher age group (>72 years). In the latter, it is significantly increased (endothelial as well as pericyte BL) compared to that in lower age group ($N = 9$ in each group; stars denote $p = 0.0001$ (A) and 0.01 (B), Student's *t*-test).

SMC of arteries (Fig. 14B), indicating that apoptosis leads to SMC degeneration in them. Positive control of retinal sections treated with DNAse showed labelling in most cellular nuclei (Fig. 14C), indicating that nuclear labelling in the vessels due to DNA damage was not artefactual. Such DNA damage may in part stem from lipid peroxidation. However, TUNEL positivity was not common in the materials examined and this may be linked to extended life-span in humans, thereby making it uncertain to find apoptotic elements in vessels in a given retina.

While lipid peroxidation was prominent in aging human retinal vessels, there was no signs of nitrosative stress in them, as understood by an absence of IR towards nitrotyrosine (Fig. 15A–D), a known marker of protein tyrosine nitration (and hence nitrosative stress).

### 3.4. Microglial reaction towards damaged vessels

Microglia were mainly distributed in the inner retina (Fig. 16A and B). In aged retinas, the Iba-1 positive microglial cells were found in the NFL and GCL (Fig. 16C–F). A few of them adhered to blood vessels of the NFL (Fig. 16C), GCL (Fig. 16D) and INL (Fig. 16G–I), these vessels were degenerative and sclerosed. Their shape appeared round and a morphology with less branches, suggesting their active nature. TEM showed microglial adherence to partially necrosed capillary wall (Fig. 16J, Supplementary Fig. 8A and B), they were identified by the presence of bent, long cisternae of rough endoplasmic reticulum (Mori and Leblond (1969) (Fig. 16J), and several lysosomes (Fig. 16J, supplementary Fig. 8B).

## 4. Discussion

The results of the present study show prominent age-related changes in the retinal vessels, which include mild to severe endothelial and pericyte degeneration, accumulations of lipofuscin granules, an apparent loss of filaments in pericytes and SMC and thickening of the capillary endothelial and pericyte BL. The thickening of the endothelial BL in retinal capillaries and arterioles of aged human (Cogan et al., 1968; Roy et al., 1994; Catita et al., 2015;

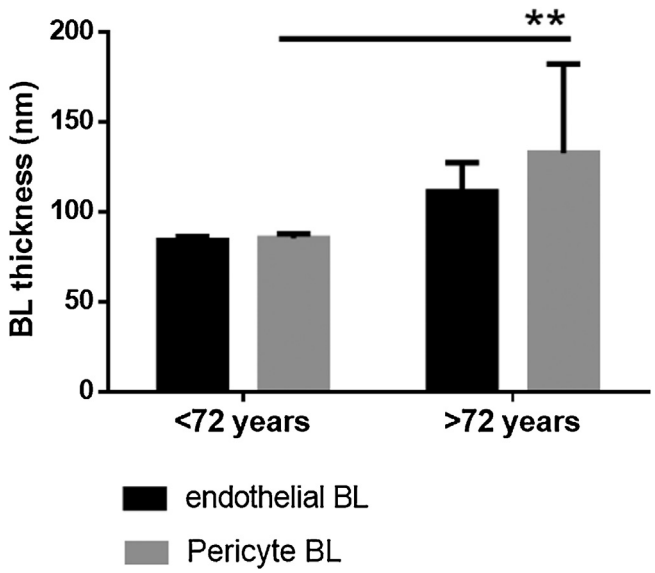


**Fig. 11.** Endothelial and pericyte BL thickness (mean ± SD) of retinal capillaries in INL was compared for lower age (<72 years) and higher age group (>72 years). In lower age group, there is no significant difference in thickness between endothelial and pericyte BL, the latter is significantly increased in higher age group compared to that in lower age group ($N = 9$ in each group; stars denote $p = 0.001$, two-way ANOVA, Post hoc Tucky's test).

Bianchi et al., 2016), ocular inflammation (Bianchi et al., 2015) and aged Wistar rats has been reported (Hughes et al., 2006). Lipofuscin accumulation in aging retinal vessels now appears to be a common feature (Kuwabara and Cogan, 1965; Catita et al., 2015), an indication of impaired lysosomal functions (Jung et al., 2007; Ma et al., 2013), leading to cellular senescence (Georgakopoulou et al. (2013). The changes in arterioles and arteries include partial damage (during the eighth decade) to loss of endothelium and SMC in the ninth decade. These led to the appearance of many acellular, sclerosed vessels in the inner retina. Earlier studies reported degeneration of pericytes, BL thickening and formation of microaneurysm as characteristic features in human diabetic retinopathy (Ashton, 1974; Garner, 1993; Frank, 1994; Stitt et al., 1995; Ejaz et al., 2008; Lechner et al., 2017; Fehér et al., 2018). Stitt et al. (1995) reported acellular, hyalinized vessels in the retina of patients with type II diabetes. Although the donor retinas examined in this study did not have any diagnosed clinical history of diabetes, it seems that factors of both aging and diabetes (e.g., free radical induced OS; Baynes, 1991; Gupta et al., 2011) exert similar effects on retinal vessels, as they age. Roy et al. (2010) found the number of acellular capillaries and pericyte loss to be significantly increased in aged and experimental diabetic rats. We found these cells to alter appreciably in their cytoskeletal make-up with aging. Pericyte changes appear somewhat late before endothelium altered significantly in the human capillaries, and this finding corroborates animal studies showing pericyte loss to be increased significantly at a relatively advanced stage of diabetes (Roy et al., 2010).

Li et al. (1997) reported apoptotic, TUNEL-positive pericytes and endothelial cells in retinal capillaries of patients with diabetes. In aging human retina, we found clear signs of apoptosis in arteries and venules (TUNEL positivity), but not in the capillaries, which could be due to the protracted life-span of humans, making it uncertain to comment when apoptosis would be prominent in retinal vessels. However, the presence of apoptotic pericytes (e.g., supplementary Fig. S3) suggests that the outer aspects of capillar-

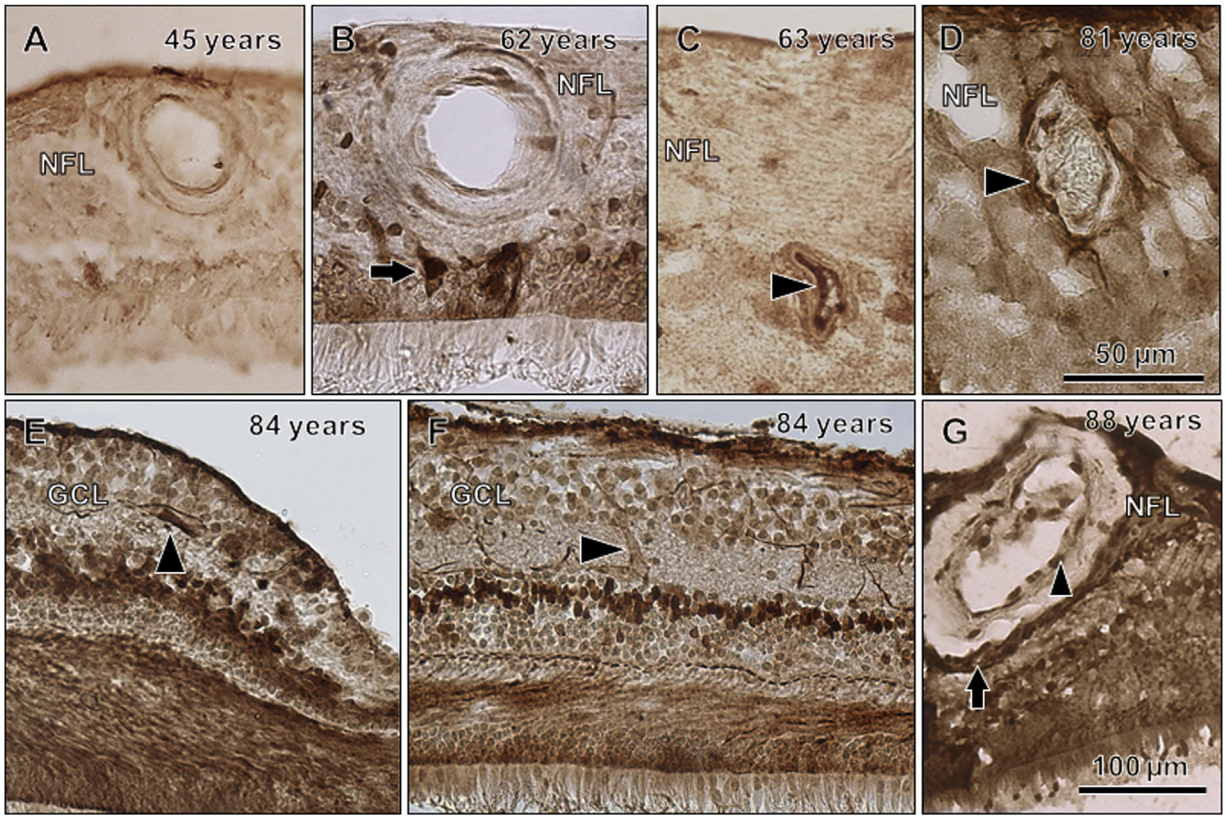


**Fig. 12.** Status of HNE-IR (arrowheads) in retinal blood vessels. (A, B) IR is absent in vessels, few INL cells show IR. (C, D) IR is seen in endothelium of one arteriole (C) and vein (D). (E, F) IR in capillaries of parafoveal GCL (E) and IPL (F) is indicated. (G) The endothelium of this sclerotic artery shows IR. Strong IR is seen in Müller cell process that invests the vessel (arrow). Donor ages are indicated on the upper right-hand corner of the images. Scale bars: 50 µm (shown in (D), also applies to (A–C)) and 100 µm (shown in (G), also applies to (E, F)).

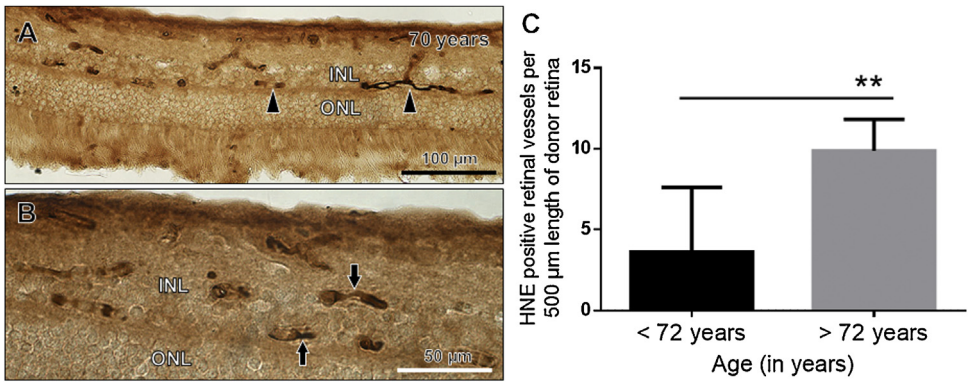


**Fig. 13.** (A) HNE-IR in aging retinal blood vessels (arrowheads). (B) Part of (A) is enlarged (from left-hand side) to show irregular contours in vessels (arrows). (C) Histogram depicting count (mean ± SD) of HNE positive retinal vessels per 500 µm length of donor retina. Compared to a lower age group (<72 years), the number of immunopositive vessels is significantly increased in the higher age group (>72 years; $p = 0.0014$, Student's $t$-test). The higher SD of the mean value in the lower age group is due to the virtual absence of IR (o) and/or few labelled vessels (1–3) in young donor retinas (35-, 45-, 54-year-old donors).

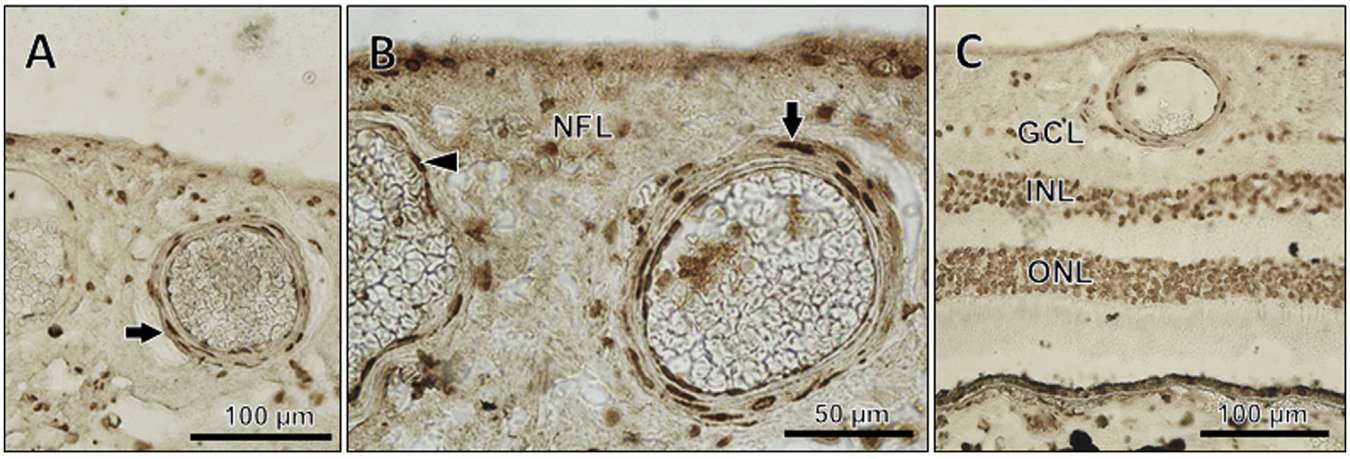


**Fig. 14.** TUNEL in retinal vessels. Labelling is present in nuclei of SMC of arteries (A, B; arrows) and endothelium of vein (B, arrowhead). Scattered nuclei in NFL also show labelling (B). (C) A positive control section showing labelling in most nuclei of GCL, INL and ONL. From 84- (A) and 88-year- (B, C) old donors.

ies undergoes significant changes and microglial presence in the capillary wall is an attempt to remodel the damaged vessel wall.

Our study revealed thickening of the endothelial and pericyte BL of capillaries, a feature that was more prominent in the advanced age group (72–94 years). BL thickening is a prominent feature in diabetic retinal vessels (Ashton, 1974; Cai and Boulton, 2002; Roy et al., 2010; Durham and Herman, 2011; Lechner et al., 2017; Fehér et al., 2018), which results from abnormal upregulation of BL components (Roy et al., 1994, 2010). Prominent thickening of the endothelial BL of capillaries located in the NFL than compared to those in outer retinal layers is earlier reported in animal models (Fischer and Gartner, 1983; Anderson et al., 1995). Our study found that not only the endothelial portion of BL, but also the portion covering the pericytes also thickened significantly in aged capillaries, a late event in capillary alterations with aging, signifying that pericytes alter later than the endothelium in aged human retinal capillaries. The thickening of the endothelial as well as pericyte BL can weaken communication between the two compartments (Stitt et al., 2016) and this may lead to the loss of capillary function.

A TEM study indicated that the close anatomical association of the retinal capillaries with Müller cells is affected in diabetic retinopathy, leading to the development of late complications (Sorrentino et al., 2016). The aging Müller cells suffer from lipid peroxidation (Nag et al., 2011), and those surrounding the retinal capillaries show numerous endosomes and lysosomes (Bianchi et al., 2016), a feature that was also seen in this study. They are thought to be involved in secreting excessive BM material in retinal capillaries not only in diabetes, but also in normal aging (Bianchi et al., 2016). Inflammatory cytokines, such as TNF-α and IL-1β (Behl et al., 2008; Bianchi et al., 2016; Roy et al., 2017) and metabolic changes in Müller cells are considered to play a pathological role in the alteration of capillary BM in aging and diabetic retinopathy (Bianchi et al., 2016; Fehér et al., 2018).

The degeneration of the medial SMC (cytoplasmic vacuolation and accumulation of membranous structures) of retinal arterioles (during fifth decade) was earlier reported by Lee et al. (1987). Although we have not seen this feature, it seems that the arterioles that bear the maximum flow pressure in vascular bed, suffers most damage earlier than the arteries. Our study showed loss of filaments and organelles in SMC and accumulation of lipofuscin granules in arteries and arterioles during the eighth and ninth decade, and the appearance of sclerosis in vessels from the ninth decade. These changes in SMC should affect the contractility of the damaged vessels and ultimately perfusion of the nearby area. The damaged cells attempt to establish anatomical connections with the healthy endothelium for possible trophic support. Endothelial cytoplasm migrating into SMC is such an attempt that helps in cross-talk between the two compartments. In this respect, it is important to emphasize that the age-related neuronal loss from the inner retina (Gao and Hollyfield, 1992; Aggarwal et al., 2007) could be due to the inherent problems with vascular perfusion, as has

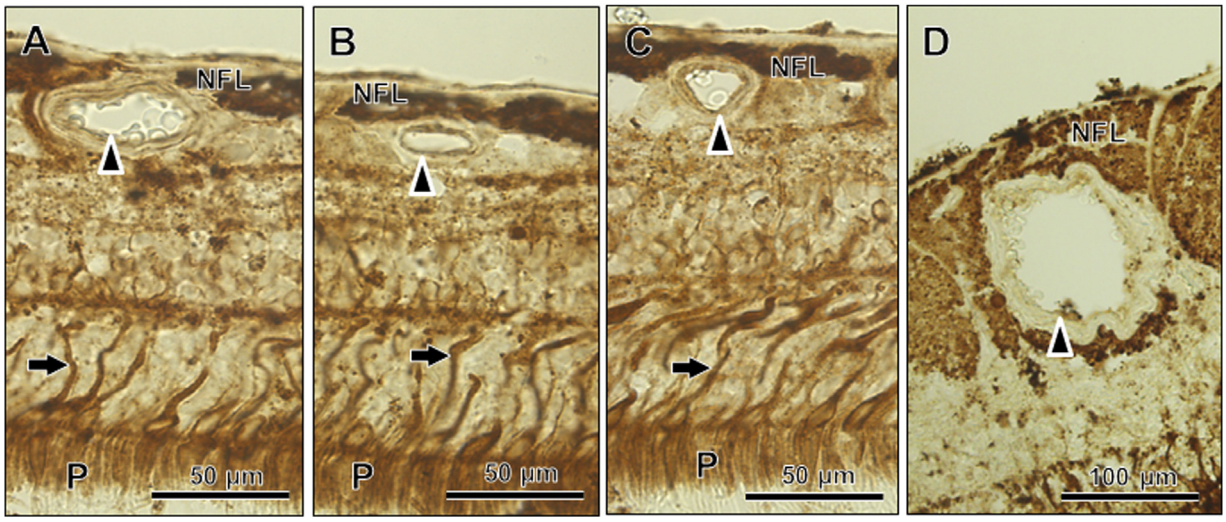


**Fig. 15.** Nitrotyrosine IR in the retina. Note absence of IR in blood vessels (arrowheads) in NFL. IR is present in NFL and photoreceptor cell axons (arrows). P, photoreceptor cells. From 64- (A–C) and 84-year-old donors.

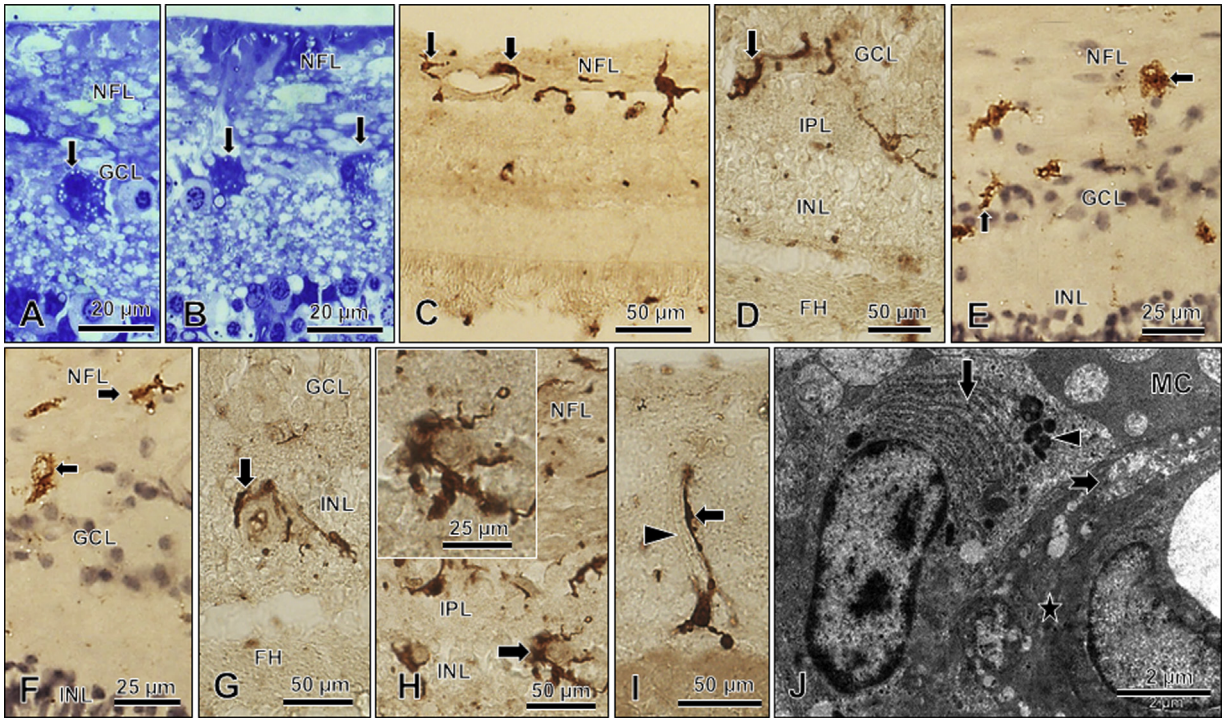


**Fig. 16.** Light micrographs (A–I) and transmission electron micrograph (J) of microglia in aged retina. (A, B) Microglia (arrows) in the GCL. (C–I) Iba-1 immunopositive microglia (arrows) in NFL (C, E, F), GCL (D) and INL (G–I). The cell displays few, short processes. Many of them adhered to vessels (arrows, C, D, G–I). (J) A microglial cell lying adherent to vessel wall (star), showing bent cisternae of rough endoplasmic reticulum (arrow), a known feature for them, and several lysosomes (arrowhead). Note that the vessel wall is necrotic (notched arrow). MC, Müller cell process. From 81-(A–C), 84-(D), 89-(E, F; counterstained with hematoxylin) and 90-year (G–J). (D) and (G) are from the macula, showing the fibre layer of Henle (FH). Inset in (H) is an enlarged view of the microglial cell indicated by arrow.

been implicated for functional alterations in retinal neurons and glia in DR (Kusuhara et al., 2018).

Studies of Roy et al. (2010, 2017) showed that retinal vascular lesions that develop in aged rats bear striking similarities to those of diabetic rats. However, the exact causes of changes in aging human retinal vessels remain unclear. Since aging human retina often accompanies OS (Nag et al., 2011, 2017, 2019), it is possible that the latter could be a culprit in vascular damage via adverse effects of free radicals on vascular endothelium and contractile elements (pericytes and SMC). Handelman and Dratz (1986) hypothesized that with exposure to oxygen free radicals may lead to excessive lipid peroxidation in retinal blood vessels, though this remained undemonstrated so far. Our study revealed that HNE-IR is significantly localized in aged capillaries and arteries, thus age-related lipid peroxidation may be involved for such damage to retinal vessels. On the contrary, nitrotyrosine IR was very insignificant in aged human retinal vessels, a fact that significantly differs from diabetic retinal vessels showing remarkable staining pattern for nitrotyrosine (Abu El-Asrar et al., 2004). Our results are in line with the fact of decreased nitric oxide production in vessel endothelium in normal aging (Hoffmann et al., 2001) and diseases (e.g., age-related macular degeneration; Bhutto et al., 2010) and that nitric oxide

is a major element in the formation of peroxynitrite, the agent responsible for inducing protein tyrosine nitration and nitrosative stress (Ischiropoulos, 1998; Szabo et al., 2007). Because HNE is marker for lipid peroxidation and generally expresses in tissues undergoing oxidative stress, it seems that human retinal vessels are susceptible to undergoing lipid peroxidation during aging, which warrants in-depth study. Inflammatory reactions often accompany aging changes, leading to alterations in retinal neurotransmitter and neurotrophin expression (Bronzetti et al., 2007), which can influence the aging retinal vessels. Studies have also shown that modified low-density lipoprotein can induce oxidative stress and endoplasmic reticulum stress, a factor that alters pericyte viability (Lupo et al., 2001) and is implicated in pericyte loss in DR (Fu et al., 2012), which is possible also with normal aging pericytes. A recent study emphasized that vascular endothelial activation could be a potent stimulator for alterations in neurovascular unit (Lenin et al., 2018). Other factors might be involved, and it is imperative to know the probable causes of those alterations and therapeutic interventions (in the form of antioxidant and nutraceutical supplements) to stop the progression of such vascular changes.

## 5. Conclusions

Vascular changes in the human retina are a prominent happening with normal progression of aging. The structural alterations of the endothelial cells and pericytes often mimic those reported for the diabetic retina and these together with a significant loss of SMC in large vessels can affect the normal functioning of the vessels. This may limit energy supply and be responsible for age-related loss of neurons of the inner retina.

## Authors' declaration

All authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

## Ethical statement

The Institute Human Ethics Committee (AIIMS, New Delhi) approved the protocols of the study (IHEC No. IEC/NP-57/2010), adhering to the tenets of Helsinki declaration. Informed consent for use of the donated eyes in research was received from relatives of the deceased.

## Conflict of interest

None.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2019.06.007.

## References

Abu El-Asrar, A.M., Meersschaert, A., Dralands, L., Missotten, L., Geboes, K., 2004. Inducible nitric oxide synthase and vascular endothelial growth factor are colocalized in the retinas of human subjects with diabetes. Eye 18, 306–313.
Aggarwal, P., Nag, T.C., Wadhwa, S., 2007. Age related decrease in rod bipolar cell density of the human retina: an immunohistochemical study. J. Biosci. 32, 293–298.
Anderson, H.R., Stitt, A.W., Gardiner, T.A., Archer, D.B., 1995. Diabetic retinopathy: morphometric analysis of basement membrane thickening of capillaries in different retinal layers within arterial and venous environments. Br. J. Ophthalmol. 79, 1120–1123.
Ashton, N., 1974. Vascular basement membrane changes in diabetic retinopathy. Br. J. Ophthalmol. 58, 344–366.
Baynes, J.W., 1991. Role of oxidative stress in development of complications in diabetes. Diabetes 40, 405–412.
Behl, Y., Krothapalli, P., Desta, T., DiPiazza, A., Roy, S., Graves, D.T., 2008. Diabetes-enhanced tumor necrosis factor-alpha production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy. Am. J. Pathol. 172, 1411–1418.
Bhutto, I.A., Baba, T., Merges, C., McLeod, D.S., Lutty, G.A., 2010. Low nitric oxide synthases (NOS) in eyes with age-related macular degeneration (AMD). Exp. Eye Res. 90, 155–167.
Bianchi, E., Ripandelli, G., Taurone, S., Feher, J., Plateroti, R., Kovacs, I., Magliulo, G., Orlando, M.P., Micera, A., Battaglione, E., Artico, M., 2016. Age and diabetes related changes of the retinal capillaries: an ultrastructural and immunohistochemical study. Int. J. Immunopathol. Pharmacol. 29, 40–53.
Bianchi, E., Ripandelli, G., Feher, J., Plateroti, A.M., Plateroti, R., Kovacs, I., Plateroti, P., Taurone, S., Artico, M., 2015. Occlusion of retinal capillaries caused by glial cell proliferation in chronic ocular inflammation. Folia Morphol. (Warsz) 74, 33–41.
Bronzetti, E., Artico, M., Kovacs, I., Felici, L.M., Magliulo, G., Vignone, D., D'Ambrosio, A., Forte, F., Di Liddo, R., Feher, J., 2007. Expression of neurotransmitters and neurotrophins in neurogenic inflammation of the rat retina. Eur. J. Histochem. 51, 251–260.
Cai, J., Boulton, M., 2002. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye (Lond.) 16, 242–260.
Catita, J., López-Luppo, M., Ramos, D., Nacher, V., Navarro, M., Carretero, A., Sánchez-Chardi, A., Mendes-Jorge, L., Rodriguez-Baeza, A., Ruberte, J., 2015. Imaging of cellular aging in human retinal blood vessels. Exp. Eye Res. 135, 14–25.
Cogan, D.G., Kuwabara, T., Friedman, T., 1968. Retinal vasculature. Microvasc. Res. 1, 115–132.
Curcio, C., 2001. Photoreceptor topography in ageing and age-related maculopathy. Eye 15, 376–383.
Curtis, T.M., Hamilton, R., Yong, P.H., McVicar, C.M., Berner, A., Pringle, R., Uchida, K., Nagai, R., Brockbank, S., Stitt, A.W., 2011. Muller glial dysfunction during diabetic retinopathy in rats is linked to accumulation of advanced glycation end-products and advanced lipoxidation end-products. Diabetologia 54, 690–698.
Durham, J.T., Herman, I.M., 2011. Microvascular modifications in diabetic retinopathy. Curr. Diab. Rep. 11, 253–264.
Ejaz, S., Chekarova, I., Ejaz, A., Sohail, A., Lim, C.W., 2008. Importance of pericytes and mechanisms of pericyte loss during diabetes retinopathy. Diab. Obes. Metabol. 10, 53–63.
Fehér, J., Taurone, S., Spoletini, M., Biró, Z., Varsányi, B., Scuderi, G., Orlando, M.P., Turchetta, R., Micera, A., Artico, M., 2018. Ultrastructure of neurovascular changes in human diabetic retinopathy. Int. J. Immunopathol. Pharmacol. 31, 394632017748841.
Fischer, F., Gartner, J., 1983. Morphometric analysis of basal laminae in rats with long term streptozotocin diabetes. II. Retinal capillaries. Exp. Eye Res. 37, 55–64.
Frank, R.N., 1994. Vascular endothelial growth factor. Its role in retinal vascular proliferative diseases. N. Engl. J. Med. 331, 1519–1520.
Fu, D., Wu, M., Zhang, J., Du, M., Yang, S., Hammad, S.M., Wilson, K., Chen, J., Lyons, T.J., 2012. Mechanisms of modified LDL-induced pericyte loss and retinal injury in diabetic retinopathy. Diabetologia 55, 3128–3140.
Gao, H., Hollyfield, J.G., 1992. Aging of the human retina. Invest. Ophthalmol. Vis. Sci. 33, 1–17.
Garner, A., 1993. Histopathology of diabetic retinopathy in man. Eye (Lond.) 7, 250–253.
Georgakopoulou, E.A., Tsimaratou, K., Evangelou, K., Fernandez Marcos, P.J., Zoumpourlis, V., Trougakos, I.P., Kletsas, D., Bartek, J., Serrano, M., Gorgoulis, V.G., 2013. Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence: a method applicable in cryopreserved and archival tissues. Aging 5, 37–50.
Gupta, S.K., Kumar, B., Nag, T.C., Agrawal, S.S., Agrawal, R., Agrawal, P., Saxena, R., Srivastava, S., 2011. Curcumin prevents experimental diabetic retinopathy in rats through its hypoglycemic, antioxidant, and anti-inflammatory mechanisms. J. Ocul. Pharmacol. Ther. 27, 123–130.
Handelman, G.J., Dratz, E.A., 1986. The role of antioxidants in retina and retinal pigment epithelium and the nature of peroxidant induced damage. Adv. Free Radic. Biol. Med. 2, 1089.
Hoffmann, J., Haendeler, J., Aicher, A., Rössig, L., Vasa, M., Zeiher, A.M., Dimmeler, S., 2001. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ. Res. 89, 709–715.
Hughes, S., Gardiner, T., Hu, P., Baxter, L., Rosinova, E., Chan-Ling, T., 2006. Altered pericyte-endothelial relations in the rat retina during aging: implications for vessel stability. Neurobiol. Aging 27, 1838–1847.
Ischiropoulos, H., 1998. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356, 1–11.

Jung, T., Bader, N., Grune, T., 2007. Lipofuscin: formation, distribution, and metabolic consequences. Ann. N.Y. Acad. Sci. 1119, 97–111.
Kusuhara, S., Fukushima, Y., Ogura, S., Inoue, N., Uemura, A., 2018. Pathophysiology of diabetic retinopathy: the old and the new. Diab. Metab. J. 42, 364–376.
Kuwabara, T., Cogan, D.G., 1965. Retinal vascular patterns. VII. Acellular change. Invest. Ophthalmol. 4, 1049–1064.
Lechner, J., O'Leary, O.E., Stitt, A.W., 2017. The pathology associated with diabetic retinopathy. Vis. Res. 139, 7–14.
Lee, W.R., Blass, G.E., Shaw, D.C., 1987. Age-related retinal vasculopathy. Eye (Lond.) 1, 296–303.
Lenin, R., Thomas, S.M., Gangaraju, R., 2018. Endothelial activation and oxidative stress in neurovascular defects of the retina. Curr. Pharm. Des. 24, 4742–4754.
Li, W., Yanoff, M., Liu, X., Ye, X., 1997. Retinal capillary pericyte apoptosis in early human diabetic retinopathy. Chin. Med. J. 110, 659–663.
Lupo, G., Anfuso, C.D., Ragusa, N., Strosznajder, R.P., Walski, M., Alberghina, M., 2001. t-Butyl hydroperoxide and oxidized low density lipoprotein enhance phospholipid hydrolysis in lipopolysaccharide-stimulated retinal pericytes. Biochim. Biophys. Acta 1531, 143–155.
Ma, W., Coon, S., Zhao, L., Fariss, R.N., Wong, W.T., 2013. A2E accumulation influences retinal microglial activation and complement regulation. Neurobiol. Aging 34, 943–960.
Minaker, K.L., 1987. Aging and diabetes mellitus as risk factors for vascular disease. Am. J. Med. 82, 47–53.
Mori, S., Leblond, C.P., 1969. Identification of microglia in light and electron microscopy. J. Comp. Neurol. 135, 57–79.
Nag, T.C., Wadhwa, S., 2012. Ultrastructure of the human retina in aging and various pathological states. Micron 43, 759–781.
Nag, T.C., Wadhwa, S., 2016. Immunolocalisation pattern of complex I–V in ageing human retina: correlation with mitochondrial ultrastructure. Mitochondrion 31, 20–32.
Nag, T.C., Wadhwa, S., Chaudhury, S., 2006. The occurrence of cone inclusions in the aging human retina and their possible effects upon vision. An electron microscope study. Brain Res. Bull. 71, 224–232.
Nag, T.C., Wadhwa, S., Alladi, P.A., Sanyal, T., 2011. Localisation of 4-hydroxy 2 nonenal immunoreactivity in ageing human retinal Müller cells. Ann. Anat. 193, 205–210.
Nag, T.C., Kumar, P., Wadhwa, S., 2017. Age related distribution of 4-hydroxy 2-nonenal immunoreactivity in human retina. Exp. Eye Res. 165, 125–135.
Nag, T.C., Kathpalia, P., Gorla, S., Wadhwa, S., 2019. Localization of nitro-tyrosine immunoreactivity in human retina. Ann. Anat. 223, 8–18.
Pizzarello, L.D., 1987. The dimensions of the problem of eye disease among the elderly. Ophthalmology 94, 1191–1195.
Pow, D.V., Sullivan, R.K., 2007. Nuclear kinesis, neurite sprouting and abnormal axonal projections of cone photoreceptors in the aged and AMD-afflicted human retina. Exp. Eye Res. 84, 850–857.
Roy, S., Maiello, M., Lorenzi, M., 1994. Increased expression of basement membrane collagen in human diabetic retinopathy. J. Clin. Invest. 93, 438–442.
Roy, S., Tonkiss, J., Roy, S., 2010. Aging increases retinal vascular lesions characteristic of early diabetic retinopathy. Biogerontology 11, 447–455.
Roy, S., Kern, T.S., Song, B., Stuebe, C., 2017. Mechanistic insights into pathological changes in the diabetic retina: implications for targeting diabetic retinopathy. Am. J. Pathol. 187, 9–19.
Sorrentino, F.S., Allkabes, M., Salsini, G., Bonifazzi, C., Perri, P., 2016. The importance of glial cells in the homeostasis of the retinal microenvironment and their pivotal role in the course of diabetic retinopathy. Life Sci. 162, 54–59.
Stitt, A.W., Gardiner, T.A., Archer, D.B., 1995. Histological and ultrastructural investigation of retinal microaneurysm development in diabetic patients. Br. J. Ophthalmol. 79, 362–367.
Stitt, A.W., Curtis, T.M., Chen, M., Medina, R.J., McKay, G.J., Jenkins, A., Gardiner, T.A., Lyons, T.J., Hammes, H.P., Simó, R., Lois, N., 2016. The progress in understanding and treatment of diabetic retinopathy. Prog. Ret. Eye Res. 51, 156–186.
Szabo, C., Ischiropoulos, H., Radi, R., 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat. Rev. Drug Discov. 6, 662–680.