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RESEARCH ARTICLE

Localization of nitro-tyrosine immunoreactivity in human retina

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ABSTRACT

Oxidative stress (OS) is associated with retinal aging and age-related macular degeneration (AMD). In both cases there are reports for the presence of markers of lipid peroxidation in retinal cells. We investigated if nitrosative stress also occurs in the human retina with aging. We examined the cellular localization of nitro-tyrosine, a biomarker of protein tyrosine nitration, in human donor retina (17–91 years; N = 15) by immunohistochemistry. Immunoreactivity (IR) to nitro-tyrosine was present in ten retinas and absent in five retinas. It was predominant in photoreceptor inner segments, cell bodies and axons. In six retinas, IR was present in abnormal, swollen axons of macular and peripheral cones. In the inner retina, weak immunoreactivity was detected in the outer and inner plexiform layer. Transmission electron microscopy revealed a variable degree of microtubule disorganization, abnormal outgrowth from the swollen macular axons (as the fibers of Henle) and few dead axons. The present study adds further evidence to the presence of aberrant photoreceptor axonal changes in the human retina and that nitro-tyrosine immunoreactivity is associated with the photoreceptor cells in select human retina.

1. Introduction

The human photoreceptor cells alter during normal aging. Both outer and inner segments show various sorts of degenerative changes (Marshall et al., 1979; Tucker, 1986; Nag et al., 2006; Pow and Sullivan, 2007; Shelley et al., 2009; Nag and Wadhwa, 2012, 2016), which often results in their death, causing a significant decline in numbers in the photoreceptor mosaic (Gartner and Henkind, 1981; Gao and Hollyfield, 1992; Curcio et al., 1994; Jackson et al., 2002; Eliasieh et al., 2007). With aging, rods die in a significantly greater number than the cones, being evident from both peripheral (Gao and Hollyfield, 1992) and macular retina (Curcio et al., 1994; Jackson et al., 2002). Cone cells, on the other hand, die sporadically, which is often detectable at a later stage of life in humans. How cone cells are altered and lost in aging is not clearly understood yet, but morphological evidence shows that such alterations may occur throughout the entire length of the cell. Concerning the human retina, earlier workers reported the cone axons to show various sorts of degenerative changes, not only in aging eyes but also in eyes with age-related macular degeneration (AMD; Pow and Sullivan, 2007; Sullivan et al., 2007; Shelley et al., 2009). We reported the human cones to show disorganized mitochondria in their ellipsoids (Nag et al., 2006; Nag and Wadhwa, 2016) and decreased expression of complex-I in the macular photoreceptors in humans above 80 years of age (Nag and Wadhwa, 2016). The reasons for those changes in the aging photoreceptor cells are unknown. Unlike other tissues, the retina has a higher oxygen demand and it is continuously exposed to visible light. It also possesses high levels of polyunsaturated fatty acids (PUFA), especially in its photoreceptor outer segments. Because of these factors the retina is prone to be injured by reactive oxygen species (ROS) and various free radicals which are produced in metabolic reactions. The retina is highly susceptible to damage by photomechanical, photothermal or photochemical mechanisms after continuous exposure to light (Mainster and Turner, 2010). When the levels of the production of ROS remain unabated by the retinal antioxidants and antioxidant enzymes (Puertas et al., 1993; De La Paz et al., 1996; Winkler et al., 1999; Beatty et al., 2000; Maeda et al., 2005; Hollyfield et al., 2008; van de Kraats et al., 2008), the result is oxidative stress, a major culprit involved in retinal diseases such as glaucoma, retinitis pigmentosa, AMD and diabetic retinopathy (Beatty et al., 2000; Komeima et al., 2006; Rogers et al., 2007; Hollyfield et al., 2008; Usui et al., 2009). In aging and AMD, oxidative stress modifies the structures of proteins and lipids (Louie et al., 2002; Ethen et al., 2007; Totan et al., 2009), which eventually can damage the normal retinal architecture.

Because aging human retina shows distinct morphological alterations and cell death, it is probable that oxidative stress plays a role in those processes (De La Paz and Anderson, 1992; Winkler et al., 1999; Handa, 2012; Handa et al., 2017). To this end, various prod-

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Table 1
Information about the eyeball donors (N = 15) and distribution of IR to nitro-tyrosine in the retina.

Age (in years) / Gender	Cause of death[a]	Delay in fixation (hours)	IR pattern
17M	Asthma	3	Absent
35M	Road-traffic accident	2	Absent
42M	Road-traffic accident	4	Absent
50M	Myocardial infarction	2	Absent
56M	Heart attack	4	Absent
62M	Heart attack	3	Photoreceptor inner segments and axons[c]
64M	Heart attack	3	Photoreceptor inner segments, cell bodies, axons[c], OPL, NFL
68M	Heart attack	3	Photoreceptor inner segments, cell bodies and macular axons[c]
73F	Heart attack	2	Photoreceptor inner segments, cell bodies and axons[c], NFL
78F	Heart attack	3	Photoreceptor inner segments, cell bodies and axons[c]
83M	Heart failure	3	Photoreceptor inner segments, cell bodies and axons[c], OPL, IPL
85M	Cardiac arrest	3	Photoreceptor inner segments, cell bodies, axons[c], OPL, IPL
88F	Cardiac arrest	4	Photoreceptor inner segments, axons[b], OPL, processes in INL, NFL
89F	Cardiac arrest	3	Photoreceptor inner segments, cell bodies and axons[c]
91M	Cardiac arrest	1	Photoreceptor inner segments, cell bodies and axons[c], OPL, IPL

[a] Information obtained from case registry; M, male; F, female.
[b] normal axons.
[c] normal as well as swollen axons, OPL, IPL, outer and inner plexiform layers; NFL, nerve fiber layer.

ucts of oxidative stress and responses to it in aging eyes have been reported (Khachik et al., 1997; Bernstein et al., 2001; Malone and Hernandez, 2007; Shen et al., 2007; Nag et al., 2011, 2017). Lipid peroxidation and protein tyrosine nitration are two major problems in many pathological conditions and aging, and in certain neurodegenerative disorders, such as multiple sclerosis, Parkinson's disease and amyotrophic lateral sclerosis (Bagasra et al., 1995; Ischiropoulos, 1998). While lipid peroxidation has been reported to be common in aging retina (De La Paz and Anderson, 1992; Puertas et al., 1993; Krohne et al., 2010; Nag et al., 2011, 2017), relatively little is known about protein tyrosine nitration in the retina (Du et al., 2002; Abu El-Asrar et al., 2004), which product nitro-tyrosine often causes nitrosative stress in the central nervous system and elsewhere (Ischiropoulos, 1998; Sawa et al., 2000; Pacher et al., 2007; Szabo et al., 2007). The formation of nitro-tyrosine begins with nitric oxide, which reacts with superoxide anion to generate peroxynitrite, a major reactive nitrogen species. This is followed by nitration of protein tyrosine residues by peroxynitrite to generate nitro-tyrosine (Ischiropoulos, 1998).

Taking previous reports into consideration (Pow and Sullivan, 2007; Shelley et al., 2009), in this study we first examined the retina by light microscopy to see if photoreceptor changes are common in aging. We found swellings in photoreceptor distal axons in few aged retinas, but not in all retinas. We then localized immunoreactivity (IR) to nitro-tyrosine, a marker of cellular nitrosative stress. Finally, we examined the fine structural alterations of photoreceptor cells with aging by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Tissue collection

Human eyes from 15 donors (Table 1) were procured from The National Eye Bank, Dr. Rajendra Prasad Center for Ophthalmic Sciences, AIIMS, New Delhi. As retrieved from medical registry, the donors had no history of ocular diseases. Consent of family members of the donors was obtained for use of the eyes in this research. The Institute Human Ethics Committee (AIIMS, New Delhi) approved the protocols of the study (As-207/2008 and IEC/NP-57/2010), adhering to the tenets of Helsinki declaration.

2.2. Tissue fixation

The left eyes were preserved in 4% paraformaldehyde for 8 h at 4 °C and used in immunohistochemistry. For TEM, the right eyes were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4 °C. After washing in buffer, the retina along with the adherent choroid and sclera was cut into a 10–12 mm long tissue strip in the temporal axis via the optic disc. Sections cut from this strip contained the macular (about 5–6 mm wide) as well as the peripheral retinal region (9–12 mm far from the macular border) via the mid-peripheral region (6–9 mm far from the macular border). The tissue pieces were put in 15–30% sucrose overnight and frozen sections of 14 μm thickness were cut. The sections were mounted onto gelatin-coated glass slides and stored at −20 °C in a refrigerator unit use.

2.3. Immunohistochemistry

Retinal sections were immunolabeled with an antibody to nitro-tyrosine (catalog number: 05–233, clone 1A6; EMD Millipore, Billerica, CA, USA). Endogenous peroxidase activity within the sections was inhibited by treatment with 0.3% hydrogen peroxide for 30 min and sections were washed. These sections were incubated in 10% horse normal serum (diluent: 0.01 M phosphate-buffer saline containing 0.5% triton X-100) and then in nitro-tyrosine antibody (mouse monoclonal, dilution: 3 μg/ml) for 48 h at 4 °C. After washing, sections were incubated in biotinylated anti-mouse IgG, followed by incubation in avidin-biotin peroxidase complex (Vectastain Elite Kit, Vector Laboratories, CA, USA). Immunoreactions were developed by treating sections in 0.06% diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich, Inc., St Louis, MO, USA) and 0.03% hydrogen peroxide. The specificity of the antibody was tested by treating retinal sections for 48 h in the primary nitro-tyrosine antibody (dilution: 3 μg/ml) that was pre-adsorbed with nitro-tyrosine control (containing nitrated proteins, catalog number: 12–354, Merck-Millipore, CA, USA; dilution: 6 μg/ml); initially both were allowed to react for 4 h in a glass tube at 4 °C and then this mixture was applied to the sections for incubation for 48 h. The rest of the steps in immunolabeling was the same as already outlined. The sections were dehydrated in ethanol and mounted in DPX. Few sections were counterstained with hematoxylin before dehydration to show the retinal layers. Also, adjacent retinal sections were stained with hematoxylin and eosin to see the cellular features, especially the photoreceptor axons. Photographs of the sections were taken under a light microscope (Leica DM6000 B) and images acquired with a digital camera, using software [Leica Application Suite, Version 3.4.1; Leica Microsystem (Switzerland)]. Brightness and contrast in those images were adjusted using Adobe Photoshop 7 (Adobe; CA, USA).

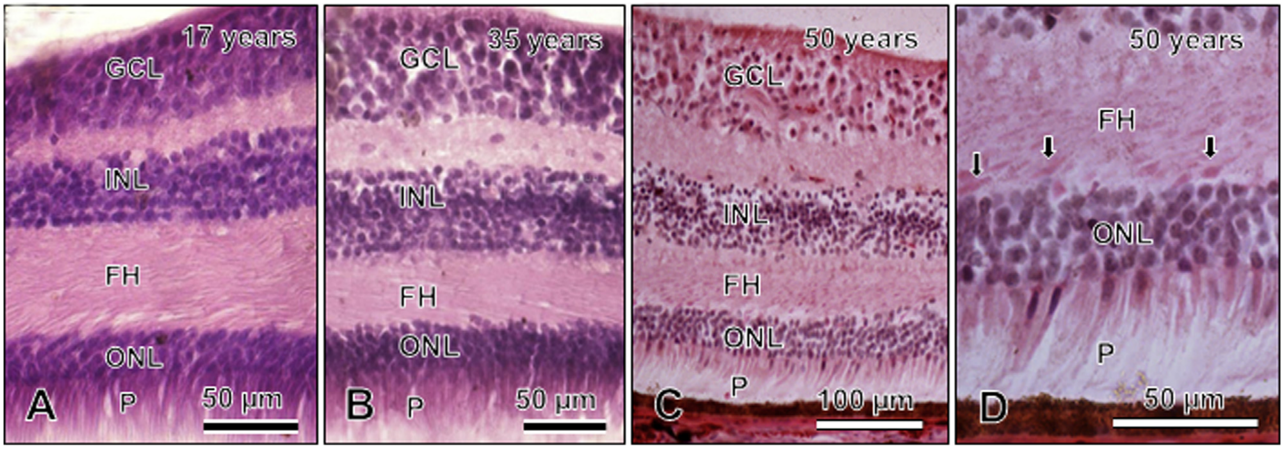

Fig. 1. Light micrographs of human retina (parafoveal region) showing the status of photoreceptor cell axons located in the fiber layer of Henle (FH). Fig. 1A and B show normal photoreceptor axons (without swelling). Fig. 1C shows swollen axons in the FH, as depicted in high magnification view of this layer in Fig. 1D (arrows). Other layers appear normal. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; P, photoreceptor cell layer. Hematoxylin and eosin stained frozen sections. Donor ages appear on the top right hand corners of the images.

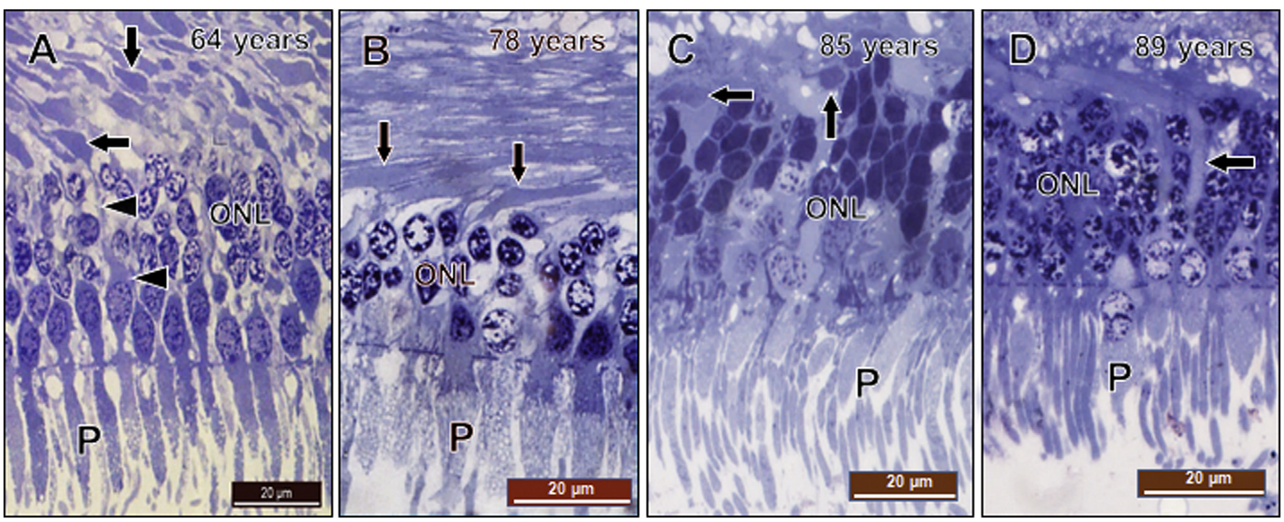

Fig. 2. Light micrographs of toluidine blue stained resin sections, showing swollen photoreceptor cell axons (arrows) in aged human retina. In Fig. 2A, a swollen distal axonal part (arrow) leading from a cone (arrowheads) is indicated. ONL, outer nuclear layer; P, photoreceptor cell layer. Donor ages appear on the top right hand corners of the images (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.4. Morphometry and quantification

Retinal sections with nitro-tyrosine positive normal and swollen photoreceptor axons were used for measurement and counting. The axons that appeared as thin, smooth fibers right from their emergence from the cell bodies were considered as normal axons, while those having conspicuous swellings along their course were considered as swollen axons. Images from five alternate sections of a retina were acquired at 40× magnification of an optical microscope and the diameter of both types of axons (N = 20) was measured in those images using ImageJ software (NIH, USA). Mean diameter of both types of axons was analyzed across the donor ages for statistical significance and increased axonal swelling, if any, with age (ordinary one way ANOVA with multiple comparisons). For counting, at 20× magnification of the microscope, the number of immunoreactive normal and swollen axons in five alternate sections from a retina was counted in a linear distance of 500 µm along the macula and adjacent part of the retina. The initial count revealed no specific variations in any region, hence data were not separated into macular versus peripheral retinal regions. The mean number (values from five sections of a donor retina) of normal and swollen axons was calculated and the percentage of swollen axons amongst the normal immunoreactive axons is shown. For statistical analysis, the data were pooled into lower age group donors (<below 80 years; 62-, 64-, 73- and 78-years) and advanced aged donors (>above 80 years; 83-, 85-, 88-, 89- and 91 years). The significance of occurrence of swollen axons was determined between the two groups (Mann-Whitney test), where p value <0.05 was considered to be statistically significant.

2.5. Transmission electron microscopy

The retina of the right eyes was separated out and chopped into 3 × 3 mm pieces from the macular and near peripheral region (4–6 mm from the macular border). The tissue samples were osmicated, dehydrated and embedded in Araldite CY212. Thick sections (0.5 µm) were stained with 1% toluidine blue and observed under an optical microscope. Images were acquired with 100X oil-immersion objective using software, as mentioned earlier. For TEM, thin sections (70–80 nm thick) were cut, contrasted with uranyl acetate and alkaline lead citrate and observed under a Tecnai G^2-20 S-Twin transmission electron microscope (Fei Company,

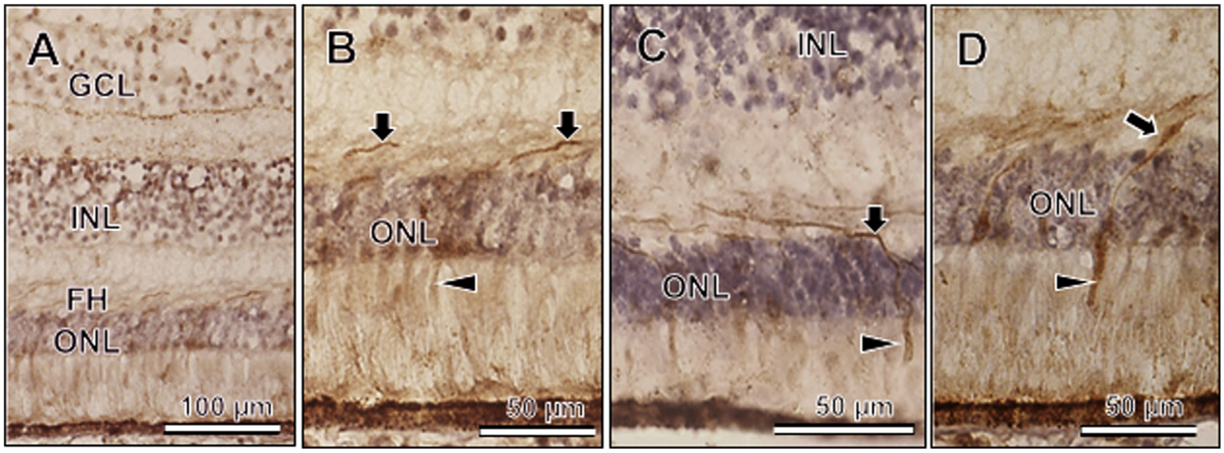

Fig. 3. Light micrographs showing IR to nitro-tyrosine in the 62-year-old donor retina. (A) Low power view of the retina. (B) Magnified view of part of the same retinal area, showing IR in two photoreceptor axons (arrows). (C, D) IR is seen in a normal and swollen axon (arrows), respectively. Arrowheads denote IR in the inner segments (B–D). GCL, ganglion cell layer; INL, inner nuclear layer; FH, fiber layer of Henle; ONL, outer nuclear layer counterstained with hematoxylin.

Eindhoven, The Netherlands). Images were digitally acquired using Digital Micrograph software (Gatan, Inc).

3. Results

3.1. Light microscopy

We mainly focused our attention on photoreceptor cells and their alterations, if any, with aging. In normal human retinas, the photoreceptor cell axons appear as thin, smooth fibers that emerge from the cell bodies and run through the outer nuclear layer (ONL) and synapse in the outer plexiform layer (OPL). In the macular region, the obliquely oriented, long photoreceptor axons are called the fibers of Henle. In young donor retinas below 50 years of age, the photoreceptors and their axons appeared to be normal (i.e., without swelling), as was found in the 17-, 35- (Fig. 1A, B) and 42-year-old (not shown) donor retinas. In the 50-year-old retina, small swellings of axons were noted in the macula (Fig. 1C, D). The retinas from the 56-, 62- (supplementary Fig.1A, B), 68- and 88-year-old donors showed normal axons (not shown), whereas the 64-year-old retina contained many prominent swollen axons (Fig. 2A). There were occasional photoreceptor axonal swellings in the 73-year and 78-year-old donor retinas (Fig. 2B; 78-year-old). Similar to the case of the 64-year-old donor, in the advanced aged retinas (83-, 85-, 89- and 91-year-old donors), the distal parts of the axons showed large swellings (Fig. 2C, D; 85-and 89-year-old), a feature that was present in photoreceptor cells of the macular as well as peripheral retina.

3.2. Pattern of expression of nitro-tyrosine IR

IR to nitro-tyrosine was absent in the 17-, 35-, 42-, 50- and 56-year-old retinas (Table 1; Supplementary Fig. 2A–D). In other retinas, IR was detected in photoreceptor inner segments, cell bodies and axons, outer and inner plexiform layer (OPL, IPL; Figs. 3–5; Supplementary Figs. 3–5), and in few cases, in the nerve fiber layer (Fig. 3B, E, Supplementary Fig. 5). The 62-year-old donor retina showed weak IR in few photoreceptor cells and their axons (normal as well as swollen; Fig. 3A–D). In the retina of the 64-year-old donor, IR was seen in normal macular (Fig. 4A) and swollen mid-peripheral axons (Fig. 4B, C). In the 68-year-old retina, occasional presence of IR in a few cones and their swollen axons was found in the macula, elsewhere the IR was absent (Supplementary Fig. 3). The 73- and 78-year-old retinas showed IR in few axonal swellings in the mid-peripheral (Fig. 4E, F; 73 years) and macular region (Fig. 4G, 78 years), otherwise the vast majority of the axons were normal (Fig. 4D; 73-years, macular and Fig.4H, I; 78-years, mid-peripheral parts). Overall, in five retinas (64-, 83-, 85-, 89- and 91-year-old), IR was found in many swollen photoreceptor axons (Fig.4B, C, 5, Supplementary Fig. 4). In the 88-year-old retina, nitro-tyrosine IR showed smooth photoreceptor axons in the macular as well as in the peripheral retina, and unidentified processes in the INL (Supplementary Fig. 5A, B).

Retinal sections treated with nitro-tyrosine antibody that was initially pre-adsorbed with nitro-tyrosine control showed no immunoreactions in any donor retinas (Fig. 6).

The mean diameter of normal and swollen axons in the donor retinas examined is shown in Fig. 7. Intergroup comparisons revealed that the swollen axons were significantly dilated from the normal axons ($P < 0.05$) in those retinas, though such swelling was not linked to aging and statistically insignificant. Fig. 8A shows the percentage of nitro-tyrosine positive swollen axons against normal axons, which varied from 2.92 to 40.81% in eight retinas. Statistical analysis revealed that the percentage of such swollen axons in the advanced aged donor retinas was not significant, compared to that in lower ages (Fig. 8B).

3.3. Ultrastructural changes in photoreceptor inner segments

TEM of photoreceptor cells showed alterations in the cone inner segments and fibers of Henle, the latter were replete with microtubules with an individual diameter of 24–25 nm. They were interleaved by dense outer processes of Müller cells, as described in literature (Hogan et al., 1971). In few donor retinas (83-, 85- and 89- year-old), there was evidence for the presence of occasional dark cone inner segments (Fig. 9A–C) containing relatively dense, shrunken mitochondria (Fig. 9E–J) other than those of the rods (Fig. 9D), and dark axonal fibers (Fig. 10A–C). The axonal microtubules altered to a variable extent; at focal points they appeared disorganized, leading to the accumulation of dense materials in them (Fig. 10D). Few of these fibers contained membrane bound vacuoles, probably of lysosomal origin (Fig. 10E, F). In other instances, the fibers of Henle showed marked dissolution of microtubules (Fig. 10G). In two maculae (83- and 85-year-old), the fibers of Henle showed irregular axonal outline (Fig. 11A, B), with a presence of outgrowths from swollen axons (Fig. 11C–F). Neurite-like extensions from dark axons were found in one retina (91-year-old; Fig. 11G).

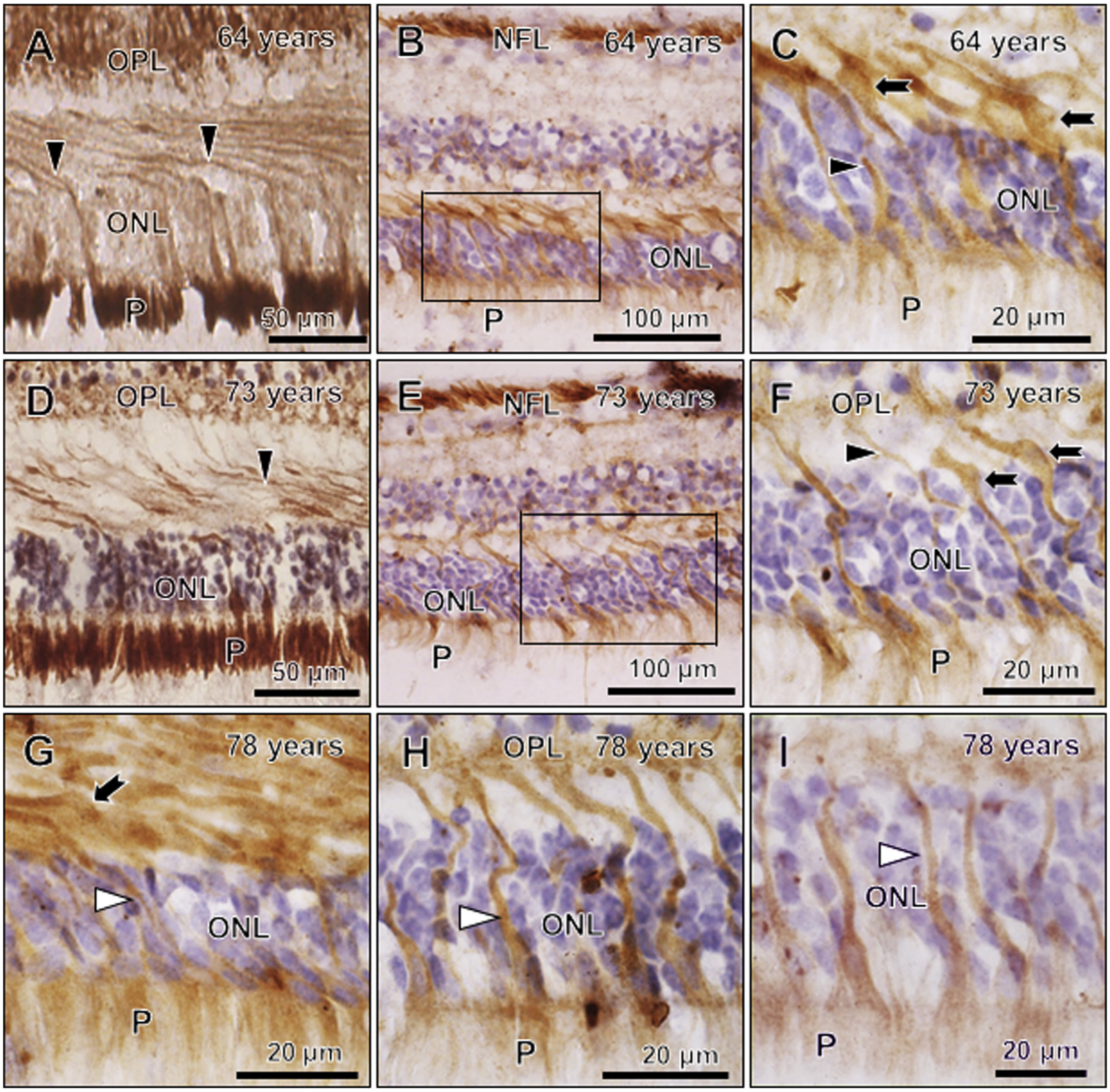

Fig. 4. Nitro-tyrosine IR in human retina. IR is present in photoreceptor inner segments (P) and axons (arrowheads). The boxed areas in Fig. B and E are enlarged to show swelling in distal axonal segments in Fig. C and F (arrows), respectively. From the macular (A, D, G) and mid-peripheral parts (B, C, E, F, H, I) of the retina. Note IR in nerve fiber layer (NFL; B, E). OPL, outer plexiform layer; ONL, outer nuclear layer; P, photoreceptor layer. Counterstained with hematoxylin. Donor ages appear on the top right hand corners of the images.

4. Discussion

In human retina, photoreceptor cell death (rod) is prominent in normal, aged persons and in those afflicted with AMD (Gao and Hollyfield, 1992; Curcio et al., 1994). Factors such as oxidative stress, nutritional status and endogenous antioxidant levels are implicated in this event (Winkler, 1999; Beatty et al., 2000; Handa et al., 2017). While lipid peroxidation has been shown to be a problem for the aging retina (De La Paz and Anderson, 1992; Puertas et al., 1993; Krohne et al., 2010; Nag et al., 2011, 2017), relatively little is known for protein tyrosine nitration, which major product nitro-tyrosine often causes nitrosative stress. Nitro-tyrosine has been identified as an indicator of cell damage and inflammation (Ceriello, 2002; Darwish et al., 2007). Its in vivo production has been detected in a large number of pathological conditions and is considered to be a marker of nitric oxide-dependent, reactive nitrogen species-induced nitrosative stress (Ischiropoulos, 1998; Sawa et al., 2000; Liu et al., 2013). To understand nitrosative stress in aging human retina, we used immunolabeling for nitro-tyrosine, which indicates sites of nitrosative damage. Our data show that protein tyrosine nitration predominantly occurs in photoreceptor inner segments and axons of the retina. Earlier studies reported nitro-tyrosine immunolabelling in the vasculature of non-diabetic and diabetic rat- (Du et al., 2002) and diabetic human retinas (Abu El-Asrar et al., 2004). Wu et al. (2005) reported tyrosine nitration in photoreceptor mitochondrial proteins in experimental uveitis. These reports and later studies (Ali et al., 2008; Liu et al., 2013) indicated the involvement of peroxynitrite and nitrated proteins as culprits in experimental uveitis and diabetic retinopathy. It is possible that cytotoxic nitrated proteins can significantly affect the normal retina, with the consequence of cell degeneration and death, especially concerning photoreceptor cells, which are common in aging and in AMD (Gao and Hollyfield, 1992; Curcio et al., 1994; Jackson et al., 2002).

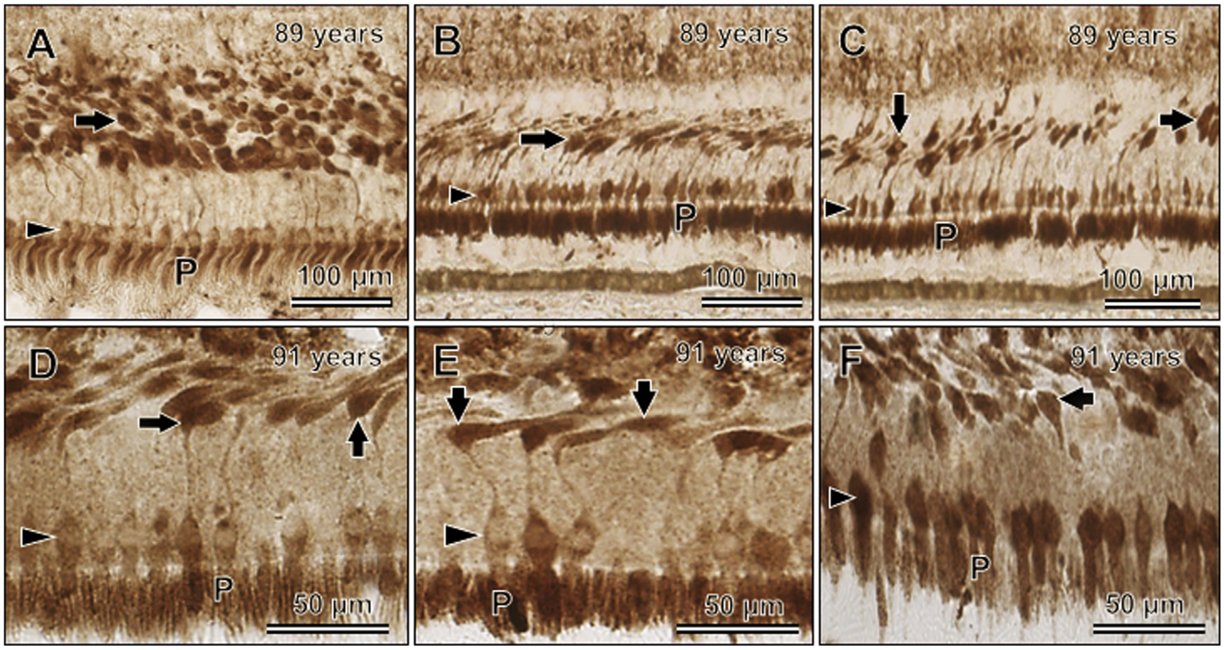

Fig. 5. Nitro-tyrosine IR in human retina. IR is present in photoreceptor inner segments (P) and in distal swollen axons (arrows) belonging to cones. Arrowheads denote the level of photoreceptor nuclei. Fig. A–C are from parafoveal (A) and perifoveal regions (B, C), respectively and Fig. D–F are from mid-peripheral region. From 89- (A–C) and 91- (D–F) year-old donors.

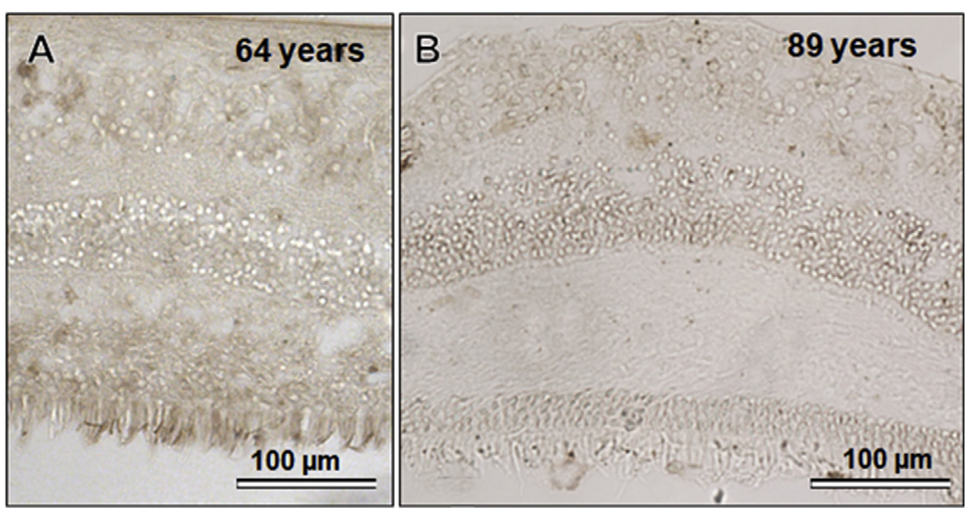

Fig. 6. Antibody specificity test. Nitro-tyrosine antibody preadsorbed with protein tyrosine nitration control failed to elicit any positive immunoreactions in photoreceptor or other cells in the retinal sections (A, B).

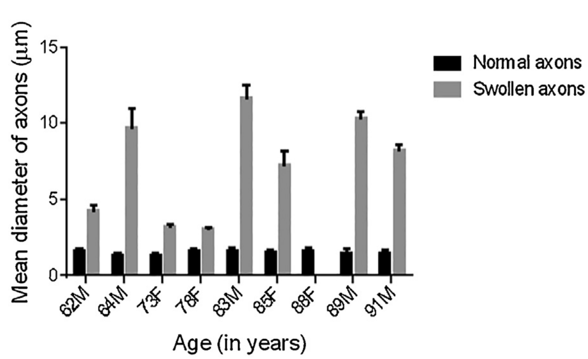

Fig. 7. Histogram of mean diameter of nitro-tyrosine positive photoreceptor axons (normal and swollen) reckoned in donor retinas. No significant variation was found in the appearance of axonal swelling with age (e.g., increase in diameter).

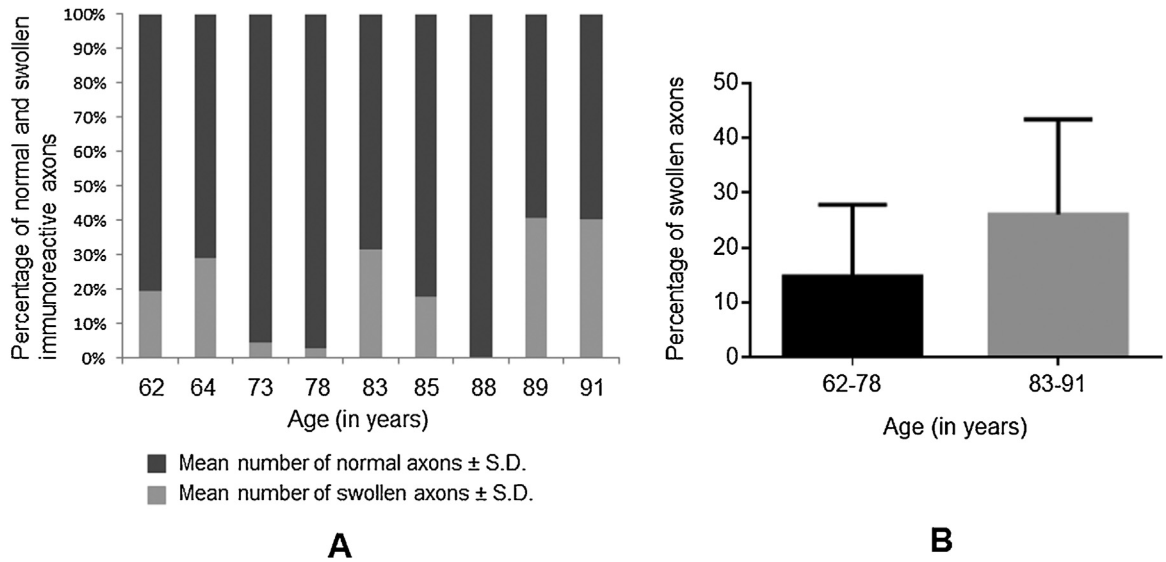

Fig. 8. A: Stacked column representation of percentage of normal and swollen, nitro-tyrosine positive photoreceptor axons in donor retinas from 62- to 91 years of age. B: Histogram of percentage of nitro-tyrosine positive photoreceptor swollen axons in lower age group (62–78 years) and advanced age group donor retinas (83–91 years of age). No significant difference was found in the percentage of swollen axons between the two groups.

Two previous studies reported cone axonal degeneration in aged eyes and in eyes with AMD (Pow and Sullivan, 2007; Shelley et al., 2009). The present study reports similar changes noted in select donor human retina. The reasons behind those changes in photoreceptors remain speculative. The fact that such changes were detected in aged retina only (>56 years of donor age) suggests a possible link with oxidative stress. The retina is rich in PUFA, has a high oxygen demand and is continuously exposed to visible light. Because of these factors, it is prone to be affected by ROS and free radicals that are generated in metabolic reactions. The observations that some donor eyes showed minimal (73- and 78 year-old) or no changes (88 year-old) in cone axons raise the possibility that individual differences in the contents of retinal anti-oxidants may reveal such apparent differences. Another variable can be related to the individual's profession that could potentially influence the physiological status of the retina and possibly changing it, as noted in this study. However, no data about the donors' profession were retrieved, and this remains an open question to see how differences in professions (e.g., one working in a job under prolonged continuous light exposure condition) could cause oxidative stress induced changes in the retina.

Earlier studies reported lipid peroxidation as an age-related event in the human retina (De La Paz and Anderson, 1992; Nag et al., 2011, 2017), wherein photoreceptor (outer segments) and Müller cells are affected. This study shows protein nitrosylation to occur in photoreceptor inner segments and axons, perhaps in situations of adverse retinal physiology. Observation of the donor retinas across the ages indicated that the IR in photoreceptor cells appeared with aging, as it was absent in young donor retinas below 56 years of age. In those retinas, the photoreceptors exhibited swellings in their distal axonal segments, which were nitro-tyrosine immunoreactive. Quantifications revealed that the number of axonal swellings showed a trend to increase in advanced aged retinas (>80 years, Fig. 8B); however, only a large cohort of samples can establish whether this could be the case or not. The findings that the swollen axons were numerous in the 64-year-old donor and only few of them were detected in the 73- and 78-year-old donor retinas deserve comments. This observation could be associated with differential reactions to the effects of oxidants (light, chemicals) by those donors, which were not age-associated. It may be that the 64-year-old donor had limited retinal antioxidant capacity compared to that in the 73- and 78-year-old donors that showed minor photoreceptor changes. This is linked to the individual redox status of reduced to oxidized glutathione (the major abundant endogenous cellular antioxidant), as well as dietary content (especially Sulphur containing amino acids) that determines the capacity to synthesize glutathione and thereby influencing its tissue content (Davidson et al., 1994; Winkler et al., 1999; Sekhar et al., 2011). It has to be emphasized here that glutathione is critically involved in counteracting nitrosative stress (Wilkins et al., 2013). So, it is probable that the donors of 64-, 83-, 89- and 91 years of age (that showed many swollen photoreceptor axons and nitro-tyrosine immunoreactivity in them) had limited protecting mechanisms against oxidative stress, thereby making the retina susceptible to undergoing damage in their photoreceptor compartments. Thus, the results of this study are of significance in the sense that cone cell death is prominent in advanced aging, beyond the ninth decade (Shelley et al., 2009), and we believe nitrosative stress (via protein tyrosine nitrosylation) may be involved in it. The exact reason for swelling of cone axons is unknown; however, this may be due to oxidative stress. It seems that some normal axons initially had undergone nitrosative injury (by protein tyrosine nitrosylation), and then became swollen due to some unknown physiological conditions, but the nitro-tyrosine marker was still retained there as changes in the nitrated proteins were not corrected by usual methods.

The degenerative axonal feature seen in aging human retina is perhaps due to their initial attack with OS. In aging, all compartments of cones are reported to be affected, most notably the axons, axon terminals and synapses (Pow and Sullivan, 2007; Shelley et al., 2009). The present study noted nitrosative stress in the photoreceptor inner segments and axons, a fact that may destabilize photoreceptor cells in certain donor retinas; this might have changed the mitochondria of the inner segments (dark and shrunken), and axonal shape, with appearance of irregular outgrowth from them.

In retinitis pigmentosa, NADPH oxidase plays a central role in cone cell death (Usui et al., 2009). Under OS, this enzyme liberates superoxide radicals that react with nitric oxide, forming peroxynitrite. The latter has a devastating effect on the cytoskeleton, altering proteins and cell signaling pathways (Szabo et al., 2007]. The degen-

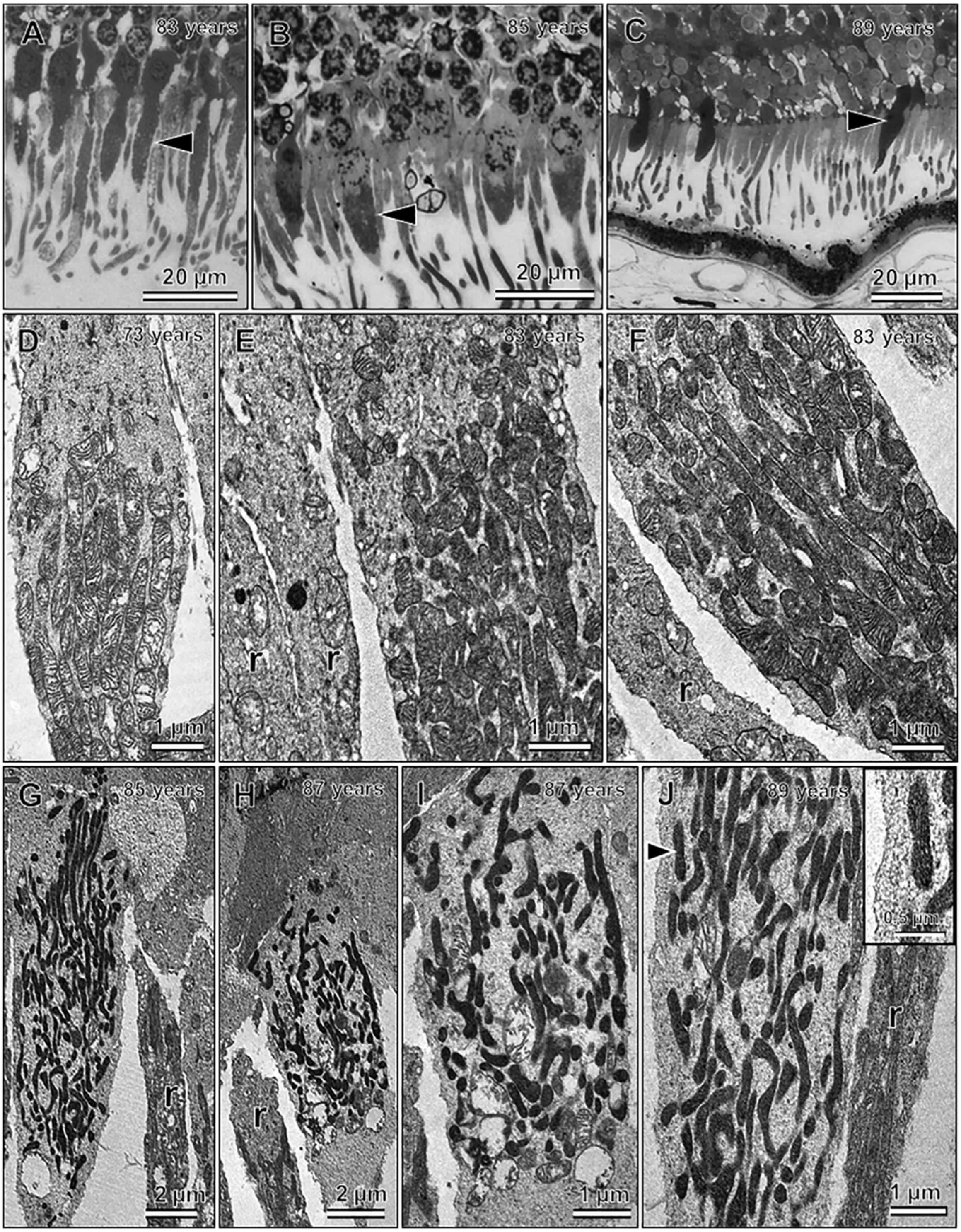

Fig. 9. Light micrographs (A–C) and transmission electron micrographs (D–J) of photoreceptor inner segments. The latter appeared dark (A–C, arrowheads) in aged retinas. In young retinas, mitochondria of cone inner segments appeared lighter (e.g., D; 73 years) than compared to those occurring in aged retinas (83–89 years), showing dense mitochondria (E–J). Rod mitochondria (r) appeared less dense (E–J) than the cone counterparts. Fig. 9I is a magnified view of Fig. 9H. Enlarged view of a dense mitochondrion is shown in the inset of Fig. 9J. Donor ages appear on the top right hand corners of the images.

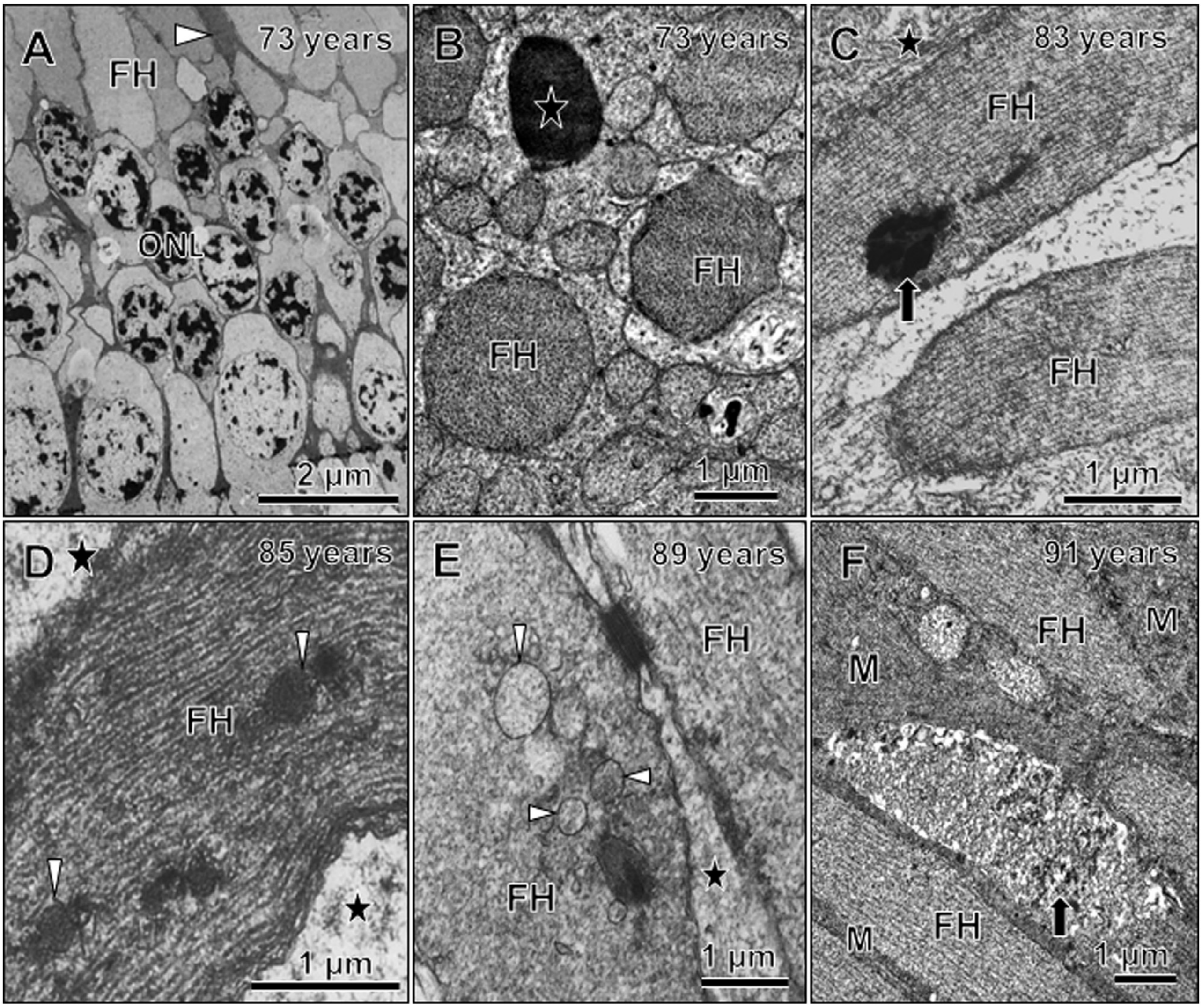

Fig. 10. Transmission electron micrographs showing alterations in the FH. (A) Low power view of FH in the parafovea. A dark fiber is indicated (arrowhead), other fibers are normal. (B) A degenerative FH (stars). (C–E) Show electron-dense material, presumably derived from degenerated microtubules ((C), arrow), and lysosomes ((D, E) arrowheads) in FH. F: Shows FH interleaved with Müller cell processes (M). In one of the fibers, there is marked dissolution of microtubules (arrow), in other FH, microtubules are intact. From 73-year (A, B), 83-year (C), 85-year (D), 89-year (E), and 91-year-old (F) donors.

erative microtubules noted in the macular axons in few aged human retina (present study) may have some links to nitrosative stress. Because antioxidants can reduce cone cell death in animal models of retinitis pigmentosa (Komeima et al., 2006), it is probable that the low antioxidant levels of the retina can trigger nitrosative stress and cone cell alterations in aged retina (see e.g., Nolan et al., 2007).

In summary, we add further evidence that in some individuals, the photoreceptor distal axons undergo characteristic swelling. Nitro-tyrosine, a biomarker of protein tyrosine nitration, can be detected by immunohistochemistry to those photoreceptor cells and their axons. This indicates that nitrosative stress may occur in human photoreceptor cells. The degeneration of photoreceptor cells, especially cones, with advanced aging (beyond the eighth decade) may in part stem from nitrosative stress that can alter the functions of mitochondria of the inner segments and destabilize the axons by altering cytoskeletal proteins.

5. Author 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, hasn't received prior publication and isn't under consideration for publication elsewhere.

6. Ethical statement

The Institute Human Ethics Committee (AIIMS, New Delhi) approved the protocols of the study (As-207/2008 and 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.

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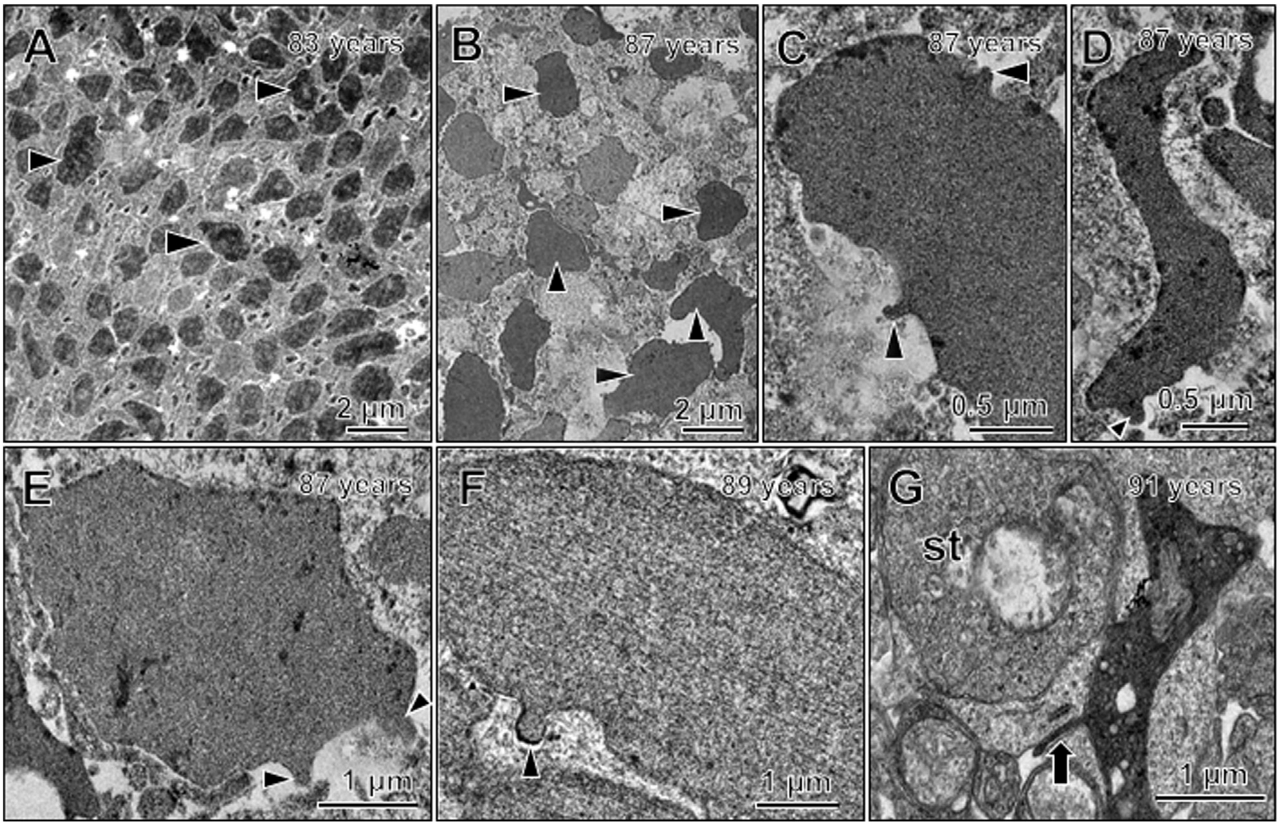

Fig. 11. Transmission electron micrographs of photoreceptor distal axons (mostly appear in cross-sectional profiles) in aged retinas. (A, B) From the FH, showing irregular outline of axons (arrowheads), more in the latter. (C–F) Outgrowths from swollen axons (arrowheads) of macular photoreceptors. (G) A neurite-like extension (arrow) from an axon located close to a synaptic terminal (st). From 83- year (A), 87-year (B–E), 89-year (F) and 91-year-old (G) donor retina.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aanat.2019.01.006.

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