Aging-related changes of optic nerve of Wistar albino rats

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Abstract Aging is a biological phenomenon that involves an increase of oxidative stress associated with gradual degradation of the structure and function of the optic nerve. Gender differences and subsequent deterioration of optic nerve are an interesting topic, especially because there is little published work concerning it. One hundred male and female Wistar albino rats' with ages 1, 6, 18, 24, and 30 months ($n=20$ equal for male and female) were used. At the time interval, optic nerve was investigated by light and transmission electron microscopy (TEM), assessments of antioxidant enzymes (catalase, superoxide dismutase, and glutathione-S-transferase), caspase 3 and 7, malondialdehyde, flow cytometry of DNA, annexin v, and CD8, immunochemistry of vascular endothelial growth factor (VEGF), CD31, and CD45, and single-strand DNA fragmentation. Light and TEM observations of the older specimens (24 and 30 months) revealed apparent deterioration of optic nerve axons,

abundant oligodendrocytes with pyknotic nuclei, swollen astrocytes, angiogenesis, vacuolar degeneration, and mitochondrial damage. Females were highly susceptible to aging processes. Concomitantly, there was a marked reduction of antioxidant's enzymes and an increase of lipid peroxidation and apoptotic markers. Old age exhibited a marked increase of G1 apoptosis, UR and LR of annexin V and CD8 as well as increased immuno-positive reaction with VEGF, CD31 and CD45. We conclude that aging contributed to an increase of oxidative stress resulting from damage of mitochondria in axons, oligodendrocytes, and astrocytes. Age-related loss of optic nerve axons is associated with multifactorial agents including reduction in antioxidant enzymes, disruption of vasculature, astrocyte, and oligodendrocyte, demyelination, and damage of mitochondria, which enhance the liberation of reactive oxygen species as assessed by an increase of apoptotic markers malondialdehyde and caspase 3 and 7.

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Introduction

Aging is a complex biological phenomenon that depends on the interaction of numerous genes, cellular pathways, and environmental risk factors. It leads to a gradual deterioration of physiological function, including impairment of vision and deterioration of the retinal cells (Harman 1981).

The optic nerve is a typical central nervous system (CNS) white matter tract and comprises the axons of retinal ganglion cells together with glia, which support them, namely, oligodendrocytes, astrocytes, microglia, and the newly described NG2-glia (Berry et al. 2002). Oligodendrocytes lie in regular interfascicular rows of five or more cells along the long axis of the nerve, interspersed with solitary astrocytes regularly spaced between the series of oligodendrocytes, which extend their primary processes orthogonally and radially to separate axon bundles into fascicles and provide the nerve with its structure (Butt et al. 1994).

CNS glia have a widespread range of physiological and pathological roles, which are the subject of numerous reviews: (1) oligodendrocytes formed the myelin sheaths that facilitate the rapid conduction of axons (Berry et al. 2002), (2) astrocytes have been considered historically as passive structural elements with multiple functions in potassium homeostasis, metabolism, axon–glial signalling, and the physiology of nodes of Ranvier and the blood–brain barrier (Rasband and Shrager 2000). In the process of aging, various organ systems are affected in the human body. Increased intraocular pressure in pigs led to swelling and damage of astrocytes (Balaratnasingam et al. 2008).

Vascular impairment of the optic nerve head may play a role in the pathogenesis of glaucoma (Groh et al. 1996) as glaucoma is more common in elderly than in younger individuals. The impact of aging on the optic nerve is of interest because it appears that aging has global effects on white matter but relatively little effect on the cortical gray matter (Peters 1999). Several studies on aging-related axonal and retinal ganglion cell loss suggested a linear process throughout adulthood (Morrison et al. 1990; Jonas et al. 1992). Luo et al. (2006) mentioned that there was a considerable reduction of optic nerve axons and astrocyte hypertrophy, especially in the center of optic nerve in old cats in comparison with young ones.

In a previous work, we reported a reduction of retinal ganglion cells (RGC) with the old age of Wistar male and female rats (El-Sayyad et al. 2013). This led our attention to these complications in the optic nerve, considering that the RGC axons concentrically organized around the optic nerve head, each of which appeared to involve both growth-promoting and growth-inhibitory guidance molecules. Together these strategies ensured proper optic nerve formation and establish the anatomical pathway for faithful transmission of information

between the retina and the brain (Oster et al. 2004); several reports indicated as well the influence of the effect of retinal ganglion cell loss on optic nerve damage (Villegas-Pérez et al. 1993; Schlamp et al. 2006).

The present study aimed to assess flow cytometry and immunohistochemical markers of apoptosis as well as biochemical and ultrastructural changes of optic nerve of albino rats of Wistar strain at 1, 6, 18, 24, and 30 months.

Materials and methods

All experiments were conducted in accordance with the Mansoura University laws for the use of animals in research and approved by the local ethical committee.

Animals

One hundred male and females Wistar albino rats (*Rattus norvegicus*) at 1, 6, 18, 24, and 30 months old were obtained from Hellwan Breeding Farm, Ministry of Health, Cairo, Egypt, and used for experimentation. During accommodation and growth, the animals were maintained in an aerated room at constant 12-h of light and dark cycle with intensity light exposure at 180–200 lx. They had free excess to standard diet and water ad libitum. The animals were sacrificed by light chloroform anesthesia at 1, 6, 18, 24, and 30 months old and dissected, with the optic nerve proper in the intraorbital segment just behind the region attached to the lamina cribrosa (optic disc) separated and subjected for investigation.

Biochemical assessments of catalase, glutathione-S-transferase, and superoxide dismutase

Fresh weights of optic tissues of aged rats were homogenated and kept in the refrigerator at -20°C . Catalase is an antioxidant enzyme involved in the detoxification of hydrogen peroxide and was determined according to Bock et al. (1980). In brief, the tissue homogenate was further diluted by phosphate buffer (pH 7.0). The reaction mixture (containing 0.05 M phosphate buffer (pH 7.0), 1.2 mM H_2O_2 , and 0.2 mL of homogenate) was allowed to stand for 30 min. The absorbance of the sample was read against distilled water at 240 nm. Glutathione-S-transferase activity was determined according to Habig et al.

(1974) by measuring the conjugation of 1-chloro-2, 4-dinitrobenzene with reduced glutathione, and absorbance was determined at 340 nm. Superoxide dismutase (SOD) activity was determined according to Niskikimi et al. (1972) based on adding 1.8 mL sodium pyrophosphate buffer, 0.5 mL of working nitroblue tetrazolium, 0.5 mL reduced nicotinamide-adenine dinucleotide, and 0.1 mL of tissue homogenate, plus 0.1 mL of freshly prepared working phenazine methosulphate. Reaction was initiated by the addition of phenazine methosulphate, and absorbance was read at 560 nm.

Lipid peroxidation end product malondialdehyde is determined according to Ohkawa et al. (1979). Two hundred microliters of the tissue homogenate supernatant were added to 100 μ L of sodium dodecyl sulfate, 750 μ L of 20 % acetic acid (pH 3.5), 750 μ L of 0.6 % thiobarbituric acid, and 300 μ L of distilled water, and the mixture was incubated at 95 °C for 60 min. After addition of 2.5 mL of butanol/pyridine (15:1) and 500 μ L of distilled water, the solution was vortexed and then centrifuged at 2,000g for 15 min. A reddish pink color developed and was analyzed at 532 nm.

Caspase 3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the sequence of events associated with apoptosis. It is determined colorimetrically by using a Stressgen Kit (Cat. No. 907-013). Retinal cells were lysed to collect their intracellular contents, and the lysate was tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the peptide can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction. Caspase 7 is a member of the caspase (cysteine aspartate protease) family of proteins and was determined by using an ELISA kit (Uscn Life Science Inc., Wuhan, China; Cat. No.: E0449Ra). The microtiter plate provided in this kit is pre-coated with an antibody specific to caspase 7. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for caspase 7. Next, avidin conjugated to horseradish peroxidase is added to each microplate well and incubated, and then a TMB substrate solution is added to each well. Only those wells that contain caspase 7, biotin-conjugated antibody, and enzyme-conjugated avidin will exhibit a color change. The

enzyme–substrate reaction is terminated by the addition of a sulfuric acid solution, and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of caspase 7 in the samples is determined by comparing the optical density of the samples to a standard curve.

Transmission electron microscopic investigations

Extra specimens were separated and fixed immediately in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After washing in the buffer, the specimens were post-fixed in a buffered solution of 1 % osmium tetroxide at 4 °C for 1.5 h, dehydrated in ascending grades of ethyl alcohol, and embedded in epoxy–resin. Ultrathin sections were cut with a diamond knife on a LKB Ultratome IV (LKB Instruments, Bromma, Sweden) and mounted on grids, stained with uranyl acetate and lead citrate, and examined under a Joel 100CX transmission electron microscope (Musashino 3-chome, Akishima, Tokyo 196-8558, Japan).

Immunostaining of vascular endothelial growth factor (VEGF), CD31 and 45

Optic nerve tissue was fixed in 10 % buffered formalin (pH 7.4) for 24–36 h before embedding in paraffin. Serial sections of 5 μ m in thickness were cut from intra-orbital optic nerve and were individually mounted onto Super Frost Plus glass slides (Fisher Thermo Scientific, Nepean, Ontario, Canada). The tissue sections were retained at normal room temperature and were processed for antigen retrieval by digestion in 0.05 % trypsin (pH 7.8) for 15 min at 37 °C and incubated with antibodies against vascular endothelial growth factor (VEGF), mouse polyclonal antibody (dilution 1:100, Cat. No. RB-9031-P) CD31, mouse polyclonal antibody (platelet endothelial cell adhesion molecule, anti-PECAM-1, dilution 1:100, Cat. No. A1-82378 (A1-82378) and CD45 (Thermo Fisher Scientific, Fremont, CA, USA; Cat. No. A1-70007) overnight at 4 °C. A horseradish peroxidase streptavidin detection system (Dako), followed with DAB plus Chromagen to detect the immunoactivity, was followed by counterstaining with hematoxylin (Sigma). Sections incubated with 1 % nonimmune serum phosphate buffer solution (PBS) solution served as negative controls. Specimens were observed with a Leica BM5000 microscope (Leica Microsystems, Wetzlar, Germany) and photographed.

Flow cytometric analysis of DNA apoptosis and annexin V and cluster of differentiation 8 (CD8)

Optic nerve samples were acquired using a FACScalibur cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a 15-mW air-cooled 488 nm argon-ion laser. FL1 (FITC) signals were detected through a 530/30-nm band pass filter; FL2 (PI) signals were detected through a 585/42 nm band pass filter. A total of 20,000 events were recorded in list mode and analyzed using the Cell Quest Pro software (Becton Dickinson) at Mansoura University Hospital. The cell populations were gated assuming the linear forward (FSC) and side-scatter (SSC) properties. Biopsies from optic nerves at the selected ages were taken, and cell suspension was prepared with Tris–EDTA buffer (pH 7.4) (Sigma-Aldrich Co.). Cell suspension was fixed in ice-cold 96–100 % ethanol (Sigma) at 4 °C overnight, centrifuged at 1,500 rpm for 10 min, and then resuspended in PBS containing 50 µg/mL propidium iodide (PI) (Sigma-Aldrich Co.). The cells were incubated at 37 °C for 30 minutes before analysis by flow cytometry. PI is excited at 488 nm and, with a relatively large Stokes shift, emits at a maximum wavelength of 617 nm. Apoptosis was indicated by the percentage of cells in G₀/G₁, S, and G₂/M phases of the cell cycle.

Also, cell apoptosis was assessed with flow cytometry using an annexin V FITC/PI staining kit (Pharmingen, Becton Dickinson Co., San Diego, CA, USA). After 48 h of transfection, we harvested cells, washed them twice in PBS (sodium chloride NaCl 40.0 g, potassium chloride KCl 1.0 g, potassium dihydrogen phosphate anhydrous KH₂PO₄ 1.0 g, disodium hydrogen phosphate anhydrous Na₂HPO₄ 4.6 g, and distilled water to make up to 51 mL; 4 °C), resuspended in the binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), stained with fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC), mixed gently and incubated for 15 min at room temperature in the dark, and then washed with binding buffer and analyzed with flow cytometry (FACS Calibar; Becton-Dickinson) using CellQuest software (Becton-Dickinson, San Jose, CA, USA).

For cluster of differentiation 8 (CD8), optic nerve specimens, suspended in RPMI solution, were centrifuged at 1,500 rpm for 5 min; the supernatant was discarded, and neuronal cells were lysed using BD pharmingen FACS Lysing Solution. The cell pellets

Table 1 Optic nerve antioxidant activities (CAT, GST, SOD), alondialdehyde (MD), and caspases (3 and 7) of 1-, 6-, 18-, 24-, and 30-month-old Wistar albino rat

	CAT		GST		SOD		MD		Caspase 3		Caspase 7	
	(nmol/min/100 mg)		(nmol/min/100 mg)		(µmol/100 mg)		(µmol/100 mg)		(µg/100 mg)		(µg/100 mg)	
	M	F	M	F	M	F	M	F	M	F	M	F
1 month	4.21±0.10	4.84±0.28	7.23±0.04	7.89±0.11	70.2±0.06	97.2±0.11	3.28±0.15	4.28±0.09	0.39±0.00	0.52±0.01	3.13±0.04	3.25±0.08
6 months	5.22±0.29	6.15±0.09	7.13±0.13	7.13±0.17	70.30±0.2	95.2±0.10	3.75±0.28	4.59±0.04	0.04±0.00	0.53±0.01	3.44±0.13	3.36±0.03
18 months	5.95±0.20	3.71±0.07	6.64±0.17	6.00±0.09	88.24±0.1	78.29±0.11	4.56±0.21	4.97±0.19	0.49±0.0	0.54±0.02	3.74±0.02	3.52±0.03
24 months	3.17±0.08	3.17±0.09	5.24±0.13	4.97±0.58	79.13±0.1	77.39±0.38	5.63±0.02	6.03±0.03	0.56±0.01	0.52±0.02	4.03±0.06	4.04±0.06
30 months	3.05±0.44	2.97±0.19	5.01±0.19	4.23±0.2	68.2±0.11	69.8±0.23	6.79±0.02	6.22±0.23	0.65±0.02	0.75±0.012	4.11±0.13	4.11±0.13
F-test	31.734		27.038		32.37		39.634		51.034		15.378	
P<	s.		s.		s.		s.		s.		s.	

Data are represented by the mean ± SE (n=5). Multivariable ANOVA SPSS

CAT catalase, GST glutathione-S-transferase, OD superoxide dismutase, MD malondialdehyde

**P<0.05 (significant; 1, 6, 18, and 30 months M and F)

were then washed and resuspended in phosphate-buffered saline. Aliquots of the cell suspension were then incubated with monoclonal antibody of CD8 (BD Biosciences, Pharmingen, FITC Mouse Anti- Human CD8 Cat. No. 555366). The neuronal cells were collected at the gate using forward and side scatter. Experiments were repeated five times for each of DNA, annexin V, and CD8 ($n=5$).

Single cell gel electrophoresis (Comet assay)

Fresh optic nerve specimens of 1-, 6-, 18-, 24, and 30-month-old rats were separated and immediately stored at -80°C . Homogenization was processed in chilled

homogenizer buffer, pH 7.5, containing 75 mM NaCl and 24 mM Na_2EDTA to obtain a 10 % tissue solution. Apotter-type was carried out and kept on freezing. Six microliters of homogenate was suspended on 0.5 % low-melting agarose and sandwiched between a layer of 0.6 % normal-melting agarose and a top layer of 0.5 % low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel layer. After the solidification of the 0.6 % agarose layer, the slides were immersed in a lyses solution (1 % sodium surcosinate, 2.5 mM NaCl, 100 mM Na_2EDTA , 10 mM Tris-HCl, 1 % tritonX-100, and 10 % dimethyl sulfoxide) at 4°C . After 1 h, the slides were placed in the electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA , pH 13) for 10 min at 4°C to allow the

Fig. 1 Photomicrographs of histological sections of 6-month-old a1 and a1 M/ a2 and a3 F, 18-month-old b and b1 M/ b2 and b3 F, 24-month-old c and c1 M/ c2 and c3 F, and 30-month-old d and d1 M/ d2 and d3 F. *a–a3* arachnoid, dura, and pia matter with well-developed neuronal cell bodies of astrocytes and oligodendrocytes and bundle of nerve fiber. *b–b3* 18 months old, showing comparative reduction of oligodendrocyte and astrocytes and slight

widening of nerve axons. *c–c3* 24 months old, showing vacuolation of nerve axons and apparent reduction of oligodendrocytes especially in females. *d–d3* 30 months old, showing abundant vacuolation of nerve axons and apparent reduction of oligodendrocyte and astrocyte. HX-E *A* arachnoid, *As* astrocyte, *BV* blood vessel, *NA* nerve axon, *O* oligodendrocyte, *ON* optic nerve, *PM* pia matter, *V* vacuole

DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 1 V/cm. The slides were neutralized with Tris–HCl buffer, pH 7.5, and stained with 20 µg/ml ethidium bromide for 10 min. Each slide was analyzed and photographed using a Leitz Orthoplan (Wetzlar, Germany) epifluorescence microscope (Sasaki et al. 1997; Robbiano et al. 2004).

Statistical analysis

Data were presented as means±standard error (SE). Statistical analysis was performed with multivariate analysis of variance using the SPSS (version 15) software package for Windows (SPSS 15.0; SPSS, Chicago, IL, USA), comparing the multivariate between each aged animal in the same group, as well as among different classes. *F*-test

was calculated and considered as statistically significant at $p<0.05$.

Results

Biochemical determination of antioxidant enzyme activities, lipid peroxidation, and caspases 3 and 7

The optic antioxidant enzyme's catalase, glutathione-*S*-transferase, and superoxide dismutase was markedly increased at 6 months old and gradually declined with aging. The activities of catalase and glutathione-*S*-transferase were affected in females more than in males. On the other hand, peroxide malondialdehyde was progressively increased during aging. The level of apoptotic markers caspase 3 and 7 was markedly increased during aging (Table 1).

Fig. 2 Transmission electron micrographs. Photomicrographs of 6-month-old (a–a3), 18-month-old (b–b3), and 30-month-old (c–c3) rats. a–a3 6 months old, showing oligodendrocyte and myelinated nerve fibers. b–b3 18 months old: b showing astrocyte with convoluted nuclear envelope, b1 showing vacuolated nerve axons, b2 and b3 showing oligodendrocyte with pyknotic

nuclei and vacuolated nerve axons. c–c3 30 months old, showing vacuolated nerve axons (c), pyknotic oligodendrocytes (c1), swollen astrocyte with damaged mitochondria and numerous lysosomes. DM damaged mitochondria, Ly lysosomes, M mitochondria, MA myelinated axon, N nucleus, PN pyknotic nuclei, VM vacuolated myelin. Lead citrate and uranyl acetate

Light and ultrastructural observations

At the light microscopic level, the optic nerve of 6- and 18-month-old rat is ensheathed by pia mater, arachnoid, and dura mater and divided into bundles by fibrous and glial septa in which run the nutrient vessels. In young and middle-aged rats, the nerve axons are seen to be closely packed and partially segregated into bundles by intervening trabeculae that contain the blood vessels. The oligodendrocytes and astrocytes were detected to be distributed throughout the core of the nerve tissues (Fig. 1, a–a3 and b–b3). At 24 and 30 months of age, there was comparative reduction of

cells and vacuolation of neuronal axons (Fig. 1, c–c3 and d–d3).

At ultrastructural level, 6-month-old rat showed wide spreading of tightly packed myelinated and non-myelinated axons of varying small sizes. The axoplasm possessed more mitochondria with a matrix of evenly spaced microtubules and neurofilaments. Numerous oligodendrocytes are detected with characteristic nuclei and cytoplasm rich in mitochondria and smooth and rough endoplasmic reticulum (Fig. 2, a–a3). Few numbers of normal blood vessels with endothelium lining cells have peculiar nuclei. Microglia cells were observed (Fig. 3, a and a2).

Fig. 3 Transmission electron micrographs. Photomicrographs of 6-month-old (a–a2), 18-month-old (b–b2), 24-month-old (c–c2), and 30-month-old (c–c3) rats. *a–a2* 6 months old, showing blood vessel with normal endothelium (*a* and *a2*) and glial cells with peculiar nuclei (*a1*). *b–b2* 18 months old, showing normal blood vessel (*b*), oligodendrocyte (*b1*), and sign of changes in glial cell and astrocyte (*b2*). *c–c2* 24 months old, showing abnormal structure of blood vessels with pyknotic endothelial

lining cells (*c* and *c1*) and glial cells with pleomorphic nuclei (*c2*). *d–d2* 30-month-old rats showing swollen blood vessel with degenerated endothelial lining cells (*d*), pyknotic oligodendrocytes (*d1*), and degenerated myelinated axons (*d2*). *AS* astrocyte, *BV* blood vessel, *DMA* demyelinated axons, *DEC* degenerated endothelial cell, *EC* endothelial cell, *GC* glia cell, *MGC* microglia cell, *ODD* oligodendrocyte, *VM*, vacuolated myelin. Lead citrate and uranyl acetate

At 18 months old, the oligodendrocytes and astrocytes were affected and possessed pyknotic nuclei with compacted heterochromatin and convoluted nuclear envelope. Demyelination and vacuolation made their first appearance within the small number of axons. Mitochondria within axoplasm showed degenerated cristae (Fig. 2, b–b3). The blood vessels become slightly swollen, and their endothelial lining cells showed nuclei with abundant heterochromatin (Fig. 3, b). Astrocyte hypertrophy was detected (Fig. 3, b1 and b2).

At 30 months old, the astrocytes become hypertrophied with detection of large cell bodies having numerous lysosomes within their cytoplasm. Many of the oligodendrocytes appeared with pyknotic nuclei. The axons showed extensive swelling and vacuolar

degeneration of the myelin sheath. The axoplasm became vacuolated and possessed widespread damage of mitochondria with missing cristae and vesiculated rough and smooth endoplasmic reticulum. There was considerable thickening of the trabeculae of fibrous connective tissue (Fig. 2, c–c3). The blood vessel appeared swollen, with damaged endothelium. Numerous small blood capillaries were detected, spreading throughout the optic tissues at 24 and 30 months old. Glia cells appeared with either pleomorphic or pyknotic nuclei (Fig. 3, c–c1 and d–d2).

Immunostaining of VEGF, CD31, and CD45

In the optic nerve of 6-month-old rats, microglia was distributed throughout the tissues in close proximity to

Fig. 4 Photomicrographs of formalin-fixed, paraffin-embedded optic nerve stained with the antibody of VEGF (a–d), CD31 (a1–d1), and CD45 (a2–d2) of 6-month-old (a–a2), 18-month-old (b–b2), 24-month-old (c–c2), and 30-month-old (d–d2) rats. Note the positive immuno-reactive VEGF of blood vessel is dense in 6 M-old and declined in old-age. Immuno-positive

cytoplasmic staining of CD31 is increased in 30 M-old-rat and confined near nuclei. CD 45 immuno staining is more dense in 30 M-old rat. Note arrays of the neuronal cell bodies of glia cells longitudinally at 24 M-old and dense positive cytoplasmic stains at 30 M-old rats.

the blood vessels and in the nerve bundles. Immuno-positive cells for VEGF, CD31, and CD45 were markedly evident in the blood vessel walls, especially in 30-month-old rats. In the 24- and 30-month-old rats, the regular array of stellar-shaped microglia was comparatively decreased (Fig. 4, a–d and c–d2).

Flow cytometry of DNA, CD8, and annexin V

From Table 2 and Fig. 5, there was a considerable increase of M1 (subG1 apoptosis) and CD8 (cluster of differentiation 8) in 24 and 30-month-old rats and a decrease in the other cell cycle phases (M2, M3, and M4). Concerning annexin V, the UR and LR showed an apparent increase during old age, and their summation, which represented apoptosis, was elevated. On the other hand, the percentages of live cells represented by LL were markedly declined.

Genomic single DNA fragmentation (Comet assay)

The genomic expression of single-cell gel electrophoresis (Comet assay) (Fig. 6) revealed increased detaching tail length and DNA concentration during the aging progress of both male and female rats, especially in 30-month-old rats compared with the normal pattern of adult and young rats (Table 3).

Discussion

Oligodendrocytes and astrocytes represent the main glia cells of optic nerve. Oligodendrocytes are responsible in the production of myelin sheaths that insulate axons, establishment of nodes of Ranvier, the sites of action potential propagation, and axonal integrity, whereas astrocytes modulate metabolism and potassium homeostasis (Butt et al. 2004). Recently, axonal myelination is precisely controlled by intercellular interactions between neurons and glia cells such as oligodendrocytes and astrocytes (Zhu et al. 2013).

From the present findings, we observed that many of the oligodendrocytes and astrocytes were abnormally damaged throughout the optic nerve tissues. The main pathological findings of oligodendrocytes include nuclear pyknosis with compacted heterochromatin materials and convoluted nuclear envelope. The astrocytes attained considerable swelling with vesiculation of RER, mitochondrial vacuolation and loss of cristae, and more increase of lysosomes. These cytological alterations reflected disruption of glia cell's metabolism and impairment of their function.

Our findings agree with many authors who outlined the contribution of oligodendrocytes and astrocytes on the function of optic nerve. Neonatal hypoxia was found to disrupt axon–oligodendrocyte integrity, leading to

Table 2 Flow cytometry of percentages of apoptosis and CD8 and annexin of optic nerve during aging of albino rats

		6 months	18 months	24 months	30 months
DNA	M1 (subG1 apoptosis)	25.09±2.87	*20.21±0.65	*69.25±4.43	*72.51±3.78
	M2 (G0/1 phase)	53.13±3.16	*62.68.56±4	*19.25±2.31	*16.52±2.56
	M3 (S phase)	9.93±1.76	5.44±1.82*	*±1.24 4.55	*0.35±2.82
	M4 (G2/M phase)	5.27±0.81	2.36±0.34*	2.20±0.27*	1.72±.14*
CD8		4.85±0.42	4.00±0.37	21.75±2.74	22.19±3.15
Annexin V	UL	0.01±00	0.02±00	0.06±00	0.71±0.03
	UR	0.73±0.02	6.67±0.84*	6.78±0.53*	7.70±1.12*
	LL	83.32±4.29	63.93±5.17*	53.76±4.85*	17.91±2.17*
	LR	15.94±1.85	29.29±3.18*	39.51±2.75*	73.68±5.73*
	Apoptosis (UR+LR)	16.67±2.13	35.96±4.71*	46.29±4.62*	81.38±5.17*

M1–M4 phases of cell cycles represented as percentages of total 100 % ($N=5$), CD8 (cluster of differentiation 8), and annexin V are represented as percentages. Apoptosis is determined by the summation of UR and LR

* $P<0.05$ (significant)

Mmonth, UL upper left, UR upper right, LL lower left, LR lower right

Fig. 5 Flow cytometry chart of CD8, DNA, and annexin V of optic nerve of rats aged 6, 18, 24, and 30 months old. CD8 and M1 DNA showing marked increase. FL1 detector fluorescence of annexin V FLTC labeled. FL2 detector fluorescence of propidium iodide stain phycoerythrin orange (PI) labeled. From annexin V chart: (1) UR showing positive annexin V and positive

propidium iodide (PI), indicating late apoptosis. (2) UL showing positive annexin V and negative PI, indicating early apoptosis. (3) LL showing negative annexin V and negative propidium iodide (PI), indicating viable cells. (4) LR showing negative annexin V and positive propidium iodide (PI), indicating necrotic cells. UR plus LR, indicating the summation of apoptosis

lasting impairment of conduction properties in the adult white matter (Ritter et al. 2013). Oligodendrocyte's losses were found to occur after axons had already degenerated (Son et al. 2010). Studies using experimental autoimmune optic neuritis models demonstrate two patterns of cell damage, including a pattern of myelin oligodendrocyte damage and a pattern of astrocyte damage (Kezuka 2013). Increase of intra-ocular pressure, which is the main phenomenon in old age (Qu et al. 2011), was parallel with the findings of Balaratnasingam et al. (2008) who reported swelling of astrocytes following an increase in intraocular pressure in pigs. Also, increased astrocyte reactivity, oligodendrocyte damage, and demyelination were reported in the optic nerve of male Wistar rat that had been diabetic for 6 weeks (Fernandez et al. 2012).

In addition, we detected widespread axonal damage involving either vacuolation or thinning of myelin

coating. Their axoplasm possessed mitochondrial damage with loss of cristae. The axonal damage reflected the damage of the oligodendrocyte and astrocytes.

A similar finding of aging-related damage of the optic nerve was reported by Dolman et al. (1980), Luo et al. (2006), and Mizrahi et al. (2011). Extensive axonal degeneration of the large heavily myelinated fibers was found in the brachial plexus from the patient with Leber hereditary optic neuropathy (Mnatsakanyan et al. 2011).

The detected damage of glia cells and demyelination was confirmed by flow cytometry assessments of the increased G1 apoptotic DNA cell cycle, UR and LR annexin V, and CD8. Annexins are a family of calcium-dependent phospholipid-binding proteins, which has a very high affinity for membranes containing the negatively charged (PS) to identify apoptosis. Dual staining with fluorescent annexin V and propidium iodide (PI)

Fig. 6 Comet assay of optic nerve of aged male (*a–a4*) and female (*b–b4*) rats. *a1* and *b1* 6-month-old male and female. *a2* and *b2* 18-month-old male and female. *a3* and *b3* 24-month-old male and female. *a4* and *b4* 30-month-old male and female. Asterisks stretched retinal cells with DNA damage which become more abundant in old age

has been used to discriminate apoptotic and necrotic cell death, in which annexin V-positive/PI-negative staining is regarded as apoptosis and PI-positive staining as necrosis (Sawai and Domae 2011).

In vitro study of elevated hydrostatic pressure was found to increase apoptosis of retinal ganglion cell-5 cell lines as assessed by flow cytometry of annexin V and intercellular reactive oxygen species as determined by flow cytometry based on 2',7'-dichlorofluorescein diacetate (Liu et al. 2012).

Demyelination of the optic nerves was observed in mice at 10 and 60 days post-infection of HSV-IL-2 (Zandian et al. 2009), which activated CD8⁺ T cells in the brain and spinal cord of ocularly infected female BALB/c mice (Osorio et al. 2005).

Furthermore, following transmission electron microscopy, we detected disorganization of the blood vessel during old age, with apparent damage of their endothelial lining cells. A striking finding is that of a widespread network of capillaries throughout the optic nerve. These were supported by increased positive immunostaining of VEGF and CD31, which reflected microvessel density. Also, decreased immunostaining of CD45 manifested disruption of vasculature and, consequently, led to hypoxic stress and decreased oxygen transmission.

Decreased oxygen supply was also reported to be involved in damage of optic nerve axons (Mizrahi et al. 2011). Vascular endothelial growth factor expression was found to precede neovascularization in the retinas and the optic nerves of humans with diabetes. Its localization to glial cells of the inner retina and the anterior optic nerve suggests a relationship to neovascularization in these sites (Amin et al. 1997). A similar increased expression of VEGF-A was reported in the optic nerve head astrocytes white light (Kernt et al. 2010).

Also, non-receptor tyrosine kinase Fyn has been found to be implicated in axon–glial signal transduction and in several cellular processes required for oligodendrocyte maturation and myelination (Krämer-Albers and White 2011). Fyn co-immunoprecipitated with CD45 from differentiating wild-type OPCs in vitro, while CD45-deficient OPCs failed to differentiate. Additionally, dysmyelination was observed in CD45-deficient mice in vivo (Nakahara et al. 2005). Increased immunopositive reaction of CD31 during old age supported the work of Liu et al. (2011) who mentioned that the number of endothelial progenitor cells in the blood increased significantly after traumatic optic nerve injury.

In addition, mitochondria are the most efficient producers of energy in the form of ATP. Energy demands of axons, placed at relatively great distances from the neuronal cell body, are met by mitochondria, which when functionally compromised produce reactive oxygen species (ROS) and, when their cellular production overwhelms the intrinsic antioxidant capacity, damage to cellular macromolecules such as DNA, proteins, and lipids ensues. Axons are made metabolically efficient by myelination, which enables saltatory conduction (Cadenas and Davies 2000; Carellia et al. 2004; Campbell and Mahad 2011). Our findings reported that apparent mitochondrial damage during old age coincides with depletion of the assayed antioxidant enzymes

Table 3 Tail length (μm) and DNA percentage (%) of optic nerve cells of 1-, 6-, 18-, 24-, and 30-month-old Wistar albino rat

	Tail length (μm)		DNA percentage (%)	
	M	F	M	F
1 month	1.495 $\pm$ 0.057	1.501 $\pm$ 0.054	0.762 $\pm$ 0.031	0.835 $\pm$ 0.083
6 months	1.524 $\pm$ 0.038*	1.754 $\pm$ 0.058*	0.935 $\pm$ 0.032*	0.840 $\pm$ 0.053*
18 months	1.954 $\pm$ 0.071**	1.542 $\pm$ 0.048*	1.257 $\pm$ 0.080**	0.738 $\pm$ 0.167*
24 months	1.754 $\pm$ 0.039**	1.952 $\pm$ 0.075**	1.430 $\pm$ 0.053**	1.611 $\pm$ 0.093**
30 months	2.910 $\pm$ 0.164**	3.550 $\pm$ 0.140**	2.510 $\pm$ 0.167**	3.050 $\pm$ 0.102**
<i>F</i> -test	65.946		69.063	
<i>P</i> <	S.		S.	

Data are represented by the mean $\pm$ SE ($n=50$)

** $P<0.05$ (significant); * $P<0.05$ (nonsignificant)

(catalase, superoxide dismutase, and glutathione-S-transferase). Mitochondria and antioxidant enzymes maintained the integrity of myelinated axons. When demyelinated, the compartmentalization of ion channels along axons is disrupted and neurodegeneration proceeds. Such oxidative stress was found to induce dysfunction of glial cells which may contribute to spreading of neuronal damage by secondary degeneration (Tezel 2006).

Liberation of superoxide ($\text{O}_2^{\bullet-}$) was found to have a negative impact on metabolic function, causing cell death of astrocytes and oligodendrocytes as well as enhanced demyelination. These were confirmed by the increase of lipid peroxidation and formation of malondialdehyde single-strand DNA damage (Comet assay) and apoptotic markers caspase 3 and 7. Also, the increase of free radicals internally damaged mitochondrial DNA (Mandavilli et al. 2000; Sawyer et al. 2001). Following assessments of monoamino-oxidase in optic nerve of old rats, Nebbioso et al. (2012) reported that age-related changes in MAO levels may be attributed to impaired energy production mechanisms.

Demyelination of optic nerve axons led to disruption of the ion channels along the axons, which proceeded to neurodegeneration. Such oxidative stress was found to induce dysfunction of glial cells, which may contribute to spreading of neuronal damage by secondary degeneration. Oxidative stress was found to promote the accumulation of advanced glycation end products during aging (Wang et al. 2009) and in glaucomatous tissues (Tezel 2006). Kanamori et al.

(2010) mentioned that superoxide was an upstream signal for retinal ganglion cell apoptosis after optic nerve injury. Early detection of axonal injury with superoxide could serve as a predictive biomarker for patients with optic neuropathy. The detected higher susceptibility of optic nerve damage of old female rats in comparison with males as determined by the assayed investigations may be attributed to the reduction of estrogen hormone during menopause. Estrogen hormone was found to play a great role in activation of both the antioxidant enzymes and mitochondrial biogenesis as mentioned by Giordano et al. (2011) following treating Leber's hereditary optic neuropathy with 17β -oestradiol.

Finally, the authors concluded that age-related loss of optic nerve axons is associated with multifactorial agents which included reduction in antioxidant enzymes, disruption of vasculature, astrocyte, and oligodendrocyte, demyelination, and damage of mitochondria, which enhance the liberation of reactive oxygen species as assessed by the increase of apoptotic markers malondialdehyde and caspase 3 and 7.

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