Realtime Imaging of Retinal Ganglion Cell Apoptosis

Maria Francesca Cordeiro,[1,2,3] Li Guo,[1,2] Katy M Coxon,[1,2] James Duggan,[1,2] Shereen Nizari,[1,2] Eduardo Normando,[1,2] Francoise Russo-Marie,[4] Clive Migdal,[3] Philip Bloom,[3] Frederick W Fitzke[2] and Stephen E Moss[4]

1. Glaucoma and Retinal Neurodegeneration Research Group, University College London Institute of Ophthalmology; 2. Visual Neurosciences, University College London Institute of Ophthalmology; 3. The Glaucoma Research Group, Western Eye Hospital, London; 4. Cell Biology Departments, University College London Institute of Ophthalmology

Abstract

Retinal ganglion cell apoptosis has long been highlighted as an important early event in glaucoma. Recent work from our group has shown that it is possible to visualise its occurrence *in vivo* using detection of apoptosing retinal cells (DARC), a recently devised non-invasive realtime imaging technique using fluorescently labelled annexin V and ophthalmoscopy. To date, DARC has been used only experimentally, but phase I clinical trials are due to start shortly in glaucoma patients. Extrapolation of these initial studies suggests that DARC may provide a new and meaningful clinical end-point in glaucoma, enabling early identification before the onset of irreversible vision loss as well as quantitative tracking of cellular degeneration and response to treatment.

Keywords

Retinal ganglion cell, apoptosis, imaging, glaucoma, detection of apoptosing retinal cells (DARC)

Disclosure: Maria Francesca Cordeiro, Frederick W Fitzke and Stephen E Moss have a patent application concerning the technology described. The remaining authors have no conflicts of interest to declare.

Received: 3 June 2010 **Accepted:** 3 September 2010 **Citation:** *European Ophthalmic Review*, 2010;4:88–91 *DOI: 10.17925/EOR.2010.04.01.88*

Correspondence: Maria Francesca Cordeiro, UCL Institute of Ophthalmology, Bath Street, London, EC1V 9EL, UK. E: M.Cordeiro@ucl.ac.uk

The retinal ganglion cell (RGC) is the key cell implicated in the development of blindness in glaucoma.[1–3] However, standard clinical tests are believed to identify visual field defects when up to as much as 40% of RGCs are lost, resulting in a potential 10-year delay in glaucoma diagnosis.[4–6] Utilising the unique optical properties of the eye, the newly developed detection of apoptosing retinal cells (DARC) technology enables direct visualisation of nerve cells dying through apoptosis, identified by fluorescent labelled annexin V. Our laboratory has assessed DARC in different retinal neuro-degenerative experimental models[7–11] and highlighted its potential in early diagnosis – in the previously regarded 'subclinical' stages of glaucoma. These studies have also demonstrated the use of DARC in assessing neuro-protective strategies.[9,10,12,13]

Principles of DARC

The process of apoptosis has been identified as the major contributory process to RGC loss in glaucoma.[1–3] Annexin V has been used for many years to identify apoptosis *in vitro*, based on its ability to bind to phosphatidylserine (PS), which becomes externalised in the outer leaflet of cells undergoing the earliest stages of apoptosis.[14] More recently, it has been used *in vivo*, particularly when tagged clinically with technetium-99m (99mTc), with applications in acute myocardial infarction and cardiac allograft rejection, ischaemic brain injury, hepatitis and lung, breast and haematological cancers.[15–21] Instead of using 99mTc, our laboratory recently showed that by tagging annexin V with a fluorescent marker, it was possible, using high-resolution imaging, to visualise and track RGC apoptosis.[8] This was originally performed using a confocal laser scanning ophthalmoscope (cLSO), with an argon laser of 488nm necessary to excite the administered annexin V-bound fluorophore, and a photodetector system with a 521nm cut-off filter to detect the fluorescent emitted light.[8,9] We subsequently used other fluorophores, but the principle of DARC remains the same.[22,23]

Until now, DARC has only been tested on experimental models.[9–11,22,23] For imaging, animals are anaesthetised, their pupils are dilated and they are positioned in front of a cLSO. Retinal images are captured using a method we have previously described,[24] from which the total number of apoptosing RGCs for each time-point *in vivo* is calculated, and an average density count per mm^2 generated (see *Figure 1*).[11] This count may be used to assess disease activity in each eye, along with the response to treatment.[9–13]

Retinal Ganglion Cell (RGC) Apoptosis and RGC Loss in Glaucoma

RGC apoptosis has been identified in clinical and experimental specimen eyes. However, until the development of DARC, evidence for apoptotic RGC death had been restricted to histological and *post mortem* analysis.[1,2,4,25,26] Nevertheless, the process of RGC apoptosis had been highlighted as one of the earliest hallmarks of the glaucomatous process.[27]

A study by Quigley et al. in experimentally induced glaucoma in monkeys showed that 4–13% of RGCs were undergoing apoptosis in early disease.[2] However, there was at least a 10-fold difference between light microscopy methods compared with terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) analysis.[2] *Post mortem* analysis of specimen eyes from patients with

glaucoma has confirmed the occurrence of RGC apoptosis,[3,28] although accurate percentage counts are not available.

Several models of ocular hypertension (OHT) have been developed in the rat, of which the technique first described by Morrison et al., and used by the current authors, has become the most popular.[29–33] The development of RGC loss in this model has been well-documented, with peak RGC loss of around 30–40% occurring at one month after intraocular pressure (IOP) elevation.[1,2,31,34–37] Within this model, RGC apoptosis occurs predominantly in the early phase of RGC loss in rat OHT, possibly as a pressure-related response.[38,39] Our studies[7–9] with DARC *in vivo*, validated histologically, showed RGC apoptosis rates of 1, 15, 13, 7 and 2% of total RGCs, with RGC losses of 17, 22, 36, 45 and 60% of the original population at two, three, four, eight and 16 weeks, respectively. This was in comparison with an optic nerve transection rat model, where RGC apoptosis levels were recorded as 0.3, 1, 8 and 3% of total RGCs, with RGC losses of 0, 3, 40 and 76% at zero, three, seven and 12 days, respectively.

In estimating the levels of RGC loss, Zeyen was the first to discuss a normal ageing rate of approximately 0.4% loss per year, compared with 4% per year due to glaucoma.[5] In the same paper, he computed that since visual field defects were only detected in glaucoma after a loss of ~40% of RGCs, standard perimetry equated to an approximate 10-year delay in diagnosis.[5] This finding is now supported by other studies.[40]

Extrapolating DARC to the Patient

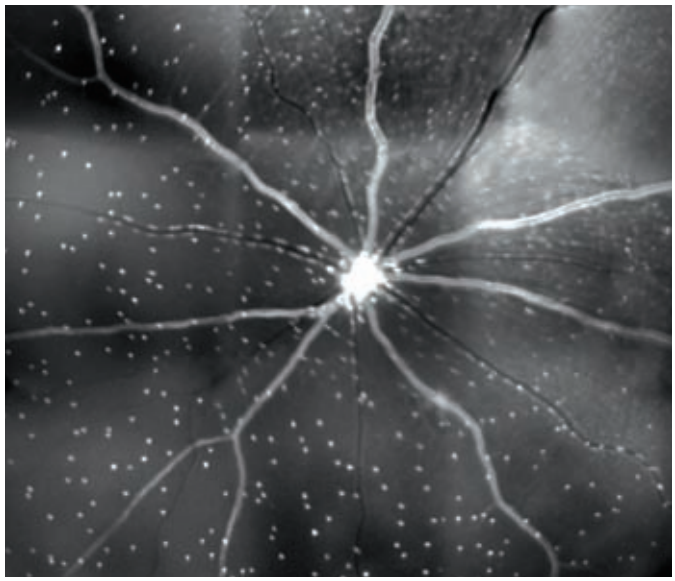
Using the rat model of experimental glaucoma, described above, we have developed an accurate profile of RGC apoptosis following surgical elevation of IOP[8] (see *Figure 2*). We applied this same profile to a hypothetical clinical situation by converting rat years into human years.[41] In this extrapolation, we assumed a sudden onset and development of the glaucomatous disease process in a 50-year-old patient. The rate of RGC loss in such a patient is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss),[5] as previously described by Zeyen. RGC numbers have been calculated in *Table 1* using these rates, but also taking into account the extrapolated profile of RGC apoptosis shown in *Figure 2*. From these, levels of RGC apoptosis per year and per day have been calculated.

Figure 3 displays the data in *Table 1* graphically. It appears that the daily count of apoptosing RGCs (the DARC count) is much greater than that in an age-matched normal eye – ranging from 50 to 400 cells per day within the first 10 years of disease. Interestingly, this 10-year period coincides exactly with the time-lag currently estimated as the delay in visual field perimetry detecting abnormalities,[5] suggesting DARC may have a role in the detection and diagnosis of early glaucoma.

Current Clinical End-points in Glaucoma

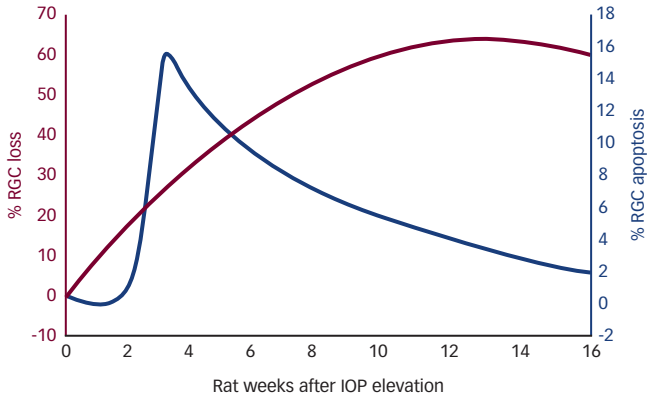
At a meeting organised by the US National Eye Institute (NEI)/US Food and Drug Administration (FDA) (13–14 March 2008, Glaucoma Clinical Drug Trial Design and End-points Symposium, Bethesda, US), a clear and unmet need in glaucoma for methods to detect this disease early, before the onset of permanent vision loss, was identified.[42] This has been further highlighted by the recent announcement of discouraging results of the first neuroprotective phase III clinical trial in glaucoma, by Allergan Inc.

Figure 1: DARC Image of the Retina of Rat Treated with Staurosporine Two Hours Previously



Each white spot is an individual retinal ganglion cell (RGC) labelled with fluorescent annexin V undergoing apoptosis, providing a snapshot of the level of RGC death at this point in time.

Figure 2: Profile of Retinal Ganglion Loss and Apoptosis in Experimental Glaucoma Model



Using an experimental model of glaucoma, we plotted the profile of retinal ganglion cell (RGC) loss (red line) and RGC apoptosis (blue line). The level of peak RGC apoptosis is at three weeks after elevated intraocular pressure (IOP), whereas maximal levels of RGC loss occur well after that, confirming previous findings that RGC apoptosis is an early marker of disease.

One of the problems outlined at the symposium was the inadequacies of single IOP measurements both as a diagnostic tool and as an index of control. This is because we now know there is a wide range of IOP in glaucoma, with low IOPs not necessarily excluding the presence of glaucomatous damage, and progressive visual field loss occurring despite normalisation of IOP in patients treated with pressure-lowering strategies.[43,44]

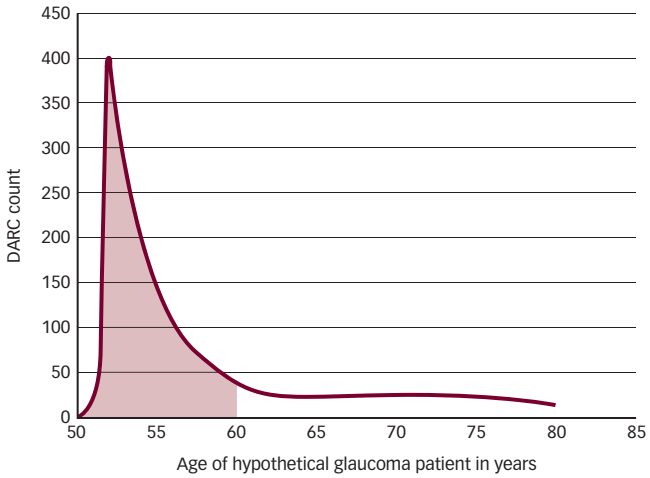
The emergence of non-IOP-lowering treatments has thus become a key research area in glaucoma, with glutamate modulation being the most advocated strategy,[13] as excitotoxicity is implicated in the development of RGC apoptosis and loss in glaucoma.[45] N-methyl-D-aspartate (NMDA) antagonists have been demonstrated to be effective in preventing neuronal degeneration in neurological disorders such as Alzheimer's disease,[46,47] but although pre-clinical demonstration of the efficacy of memantine was encouraging,[48,49] the phase III clinical trial of primary open-angle glaucoma (POAG) patients

Table 1: Rate of Retinal Ganglion Cell Loss and Apoptosis in Hypothetical Glaucoma Patient

	Hypothetical Patient Age (years)*							
	50	51	52	53	55	60	70	80
Normal RGC loss/year (x 10^3)	1,000	996	992	988	980	961	923	887
Glaucoma RGC loss/year (x 10^3)	1,000	960	922	885	815	665	442	290
Glaucoma RGC apoptosis/year	3,000	10,934	142,265	114,251	54,524	12,964	8,619	5,730
Glaucoma RGC apoptosis/day	8	30	390	313	149	35	24	16

**Glaucoma onset at 50 years of age.*
The rate of retinal ganglion cell (RGC) loss in glaucoma is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss),[5] as previously described by Zeyen. RGC numbers have been calculated using these rates, but also taking into account the extrapolated profile of RGC apoptosis shown in Figure 2, *in a hypothetical clinical situation of a patient with sudden onset of glaucoma at 50 years of age with the same profile of RGC apoptosis as the rat ocular hypertension model. Predicted levels of RGC apoptosis per year and per day are calculated, as shown.*

Figure 3: Predicted DARC Count in Hypothetical Glaucoma Patient



This graphical display of data from Table 1 *shows the predicted DARC count (retinal ganglion cell [RGC] apoptosis count/day) in the hypothetical clinical situation of a patient with sudden onset of glaucoma at 50 years of age. The maximal expected DARC count occurs within 10 years of the onset of disease (shaded area), and coincides exactly with the time-lag currently estimated as the delay in visual field perimetry detecting abnormalities.[5]*

was not. Although the full results have not yet been published, poor end-points may have been a contributory factor – IOP could not be used, so visual fields and optic disc changes were utilised and may have accounted for the long period of follow-up (>5 years) necessary for this trial.

Our group has used DARC to test of the efficacy of neuroprotective treatments in several models of glaucoma.[9,10,13,50] In fact, potentially the most immediate benefit of DARC will be in its application to directly monitor the effects of therapy in glaucoma. Glutamate modulation is not the only mode of neuroprotection, and DARC has been used to assess new strategies, such as those targeting the Alzheimer's protein beta-amyloid.[10]

Into the Future with DARC

We believe that DARC should provide a snapshot of the number of apoptosing RGC at any one time in patients. As such, it is hoped the DARC count (see *Figure 3*) will provide a new end-point in glaucoma. However, only the planned large population-based clinical studies will establish the DARC count in relation to glaucoma and the normal ageing process in order to validate the estimates above. It will also permit the investigation of whether a specific pattern of apoptosis occurs, as we postulate it will be along the pathway of retinal nerve fibres, with an increased probability of detecting focal areas of increased DARC activity in the papillo-macular bundle.

As DARC enables direct observation of single nerve cell apoptosis in experimental neurodegeneration, we are also keen to assess its use in combination with other spectrally distinct cell markers. This should permit investigation of fundamental disease mechanisms and the evaluation of interventions with clinical applications. Furthermore, as we and others have advocated, as the retina is increasingly implicated in a variety of neurodegenerative conditions,[51] we believe that investigation of such mechanisms within the eye may shed light on mechanisms underlying neurodegeneration within the brain.

DARC may thus provide a powerful new clinical tool with which to diagnose and identify patients with early glaucoma, before they lose vision. It may also dramatically reduce the duration of glaucoma clinical studies, which currently have to use visual field status as a key end-point and determinant of outcome. In clinics, it could provide a real-time, more rapid and objective method by which to monitor patients. Finally, it may also serve as a new method of assessing central nervous system (CNS) degeneration. As we await the results of the phase 1 clinical trial at the Western Eye Hospital in London, we all hope that DARC may provide the new end-point that we so clearly need in glaucoma. ■

Maria Francesca Cordeiro is a clinician scientist with a specialist interest in the field of glaucoma and neurodegeneration. She heads the Glaucoma and Retinal Neurodegeneration Research Group at the Institute of Ophthalmology, University College London, and is an Honorary Consultant Ophthalmologist at the Western Eye Hospital, St Mary's NHS Trust in London. Dr Cordiero's research focus is the molecular mechanisms involved in either the treatment or pathogenesis of retinal neurodegenerative diseases, including glaucoma, Alzheimer's and diabetes. The aims of her group's work are to establish new methods of diagnosis of early disease to avoid disability, identify early markers of cell processes in neurodegenerative disease and investigate therapeutic approaches to their treatment. She has investigated novel and translational approaches to these problems, and has received a number of awards for her work including the 2005 Lewis Rudin Glaucoma Prize from the New York Academy of Medicine, the 2000 International Glaucoma Review Award and the 1998 Moorfields Research Gold Medal. Dr Cordiero graduated in medicine from St Bartholomew's Hospital University of London in 1987 and completed training in general and surgical ophthalmology at Moorfield's Eye and St Thomas' Hospitals in London in 2003.

1. Garcia-Valenzuela E, Shareef S, Walsh J, Sharma SC, Programmed cell death of retinal ganglion cells during experimental glaucoma, *Exp Eye Res*, 1995;61:33–44.
2. Quigley HA, Nickells RW, Kerrigan LA, et al., Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis, *Invest Ophthalmol Vis Sci*, 1995;36:774–86.
3. Kerrigan LA, Zack DJ, Quigley HA, et al., TUNEL-positive ganglion cells in human primary open-angle glaucoma, *Arch Ophthalmol*, 1997;115:1031–5.
4. Kerrigan-Baumrind L, Quigley H, Pease M, et al., Number of ganglion cells in glaucoma eyes compared with threshold visual field tests in the same persons, *Invest Ophthalmol Vis Sci*, 2000;41:741–8.
5. Zeyen T, Target pressures in glaucoma, *Bull Soc Belge Ophtalmol*, 1999;274:61–5.
6. Harwerth RS, Crawford ML, Frishman LJ, et al., Visual field defects and neural losses from experimental glaucoma, *Prog Retin Eye Res*, 2002;21:91–125.
7. Guo L, Moss SE, Alexander RA, et al., Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix, *Invest Ophthalmol Vis Sci*, 2005;46:175–82.
8. Cordeiro MF, Guo L, Luong V, et al., Real-time imaging of single nerve cell apoptosis in retinal neurodegeneration, *Proc Natl Acad Sci U S A*, 2004;101:13352–6.
9. Guo L, Salt TE, Maass A, et al., Assessment of neuroprotective effects of glutamate modulation on glaucoma-related retinal ganglion cell apoptosis *in vivo*, *Invest Ophthalmol Vis Sci*, 2006;47:626–33.
10. Guo L, Salt TE, Luong V, et al., Targeting amyloid-beta in glaucoma treatment, *Proc Natl Acad Sci U S A*, 2007;104: 13444–9.
11. Maass A, von Leithner PL, Luong V, et al., Assessment of Rat and Mouse RGC Apoptosis Imaging in Vivo with Different Scanning Laser Ophthalmoscopes, *Curr Eye Res*, 2007;32:851–61.
12. Guo L, Cordeiro MF, Assessment of neuroprotection in the retina with DARC, *Prog Brain Res*, 2008;173:437–50.
13. Cheung W, Guo L, Cordeiro MF, Neuroprotection in glaucoma: drug-based approaches, *Optom Vis Sci*, 2008;85:406–16.
14. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J Immunol Methods*, 1995;184:39–51.
15. Green AM, Steinmetz ND, Monitoring apoptosis in real time, *Cancer J,* 2002;8:82–92.
16. Flotats A, Carrio I, Non-invasive in vivo imaging of myocardial apoptosis and necrosis, *Eur J Nucl Med Mol Imaging*, 2003;30:615–30.
17. Narula J, Acio ER, Narula N, et al., Annexin-V imaging for noninvasive detection of cardiac allograft rejection, *Nat Med*, 2001;7:1347–52.
18. Zhao M, Beauregard DA, Loizou L, et al., Non-invasive detection of apoptosis using magnetic resonance imaging and a targeted contrast agent, *Nat Med*, 2001;7:1241–4.
19. Blankenberg FG, Strauss HW, Will imaging of apoptosis play a role in clinical care? A tale of mice and men, *Apoptosis*, 2001;6:117–23.
20. Mountz JD, Hsu HC, Wu Q, et al., Molecular imaging: new applications for biochemistry, *J Cell Biochem Suppl*, 2002;39:162–71.
21. Reutelingsperger CP, Dumont E, Thimister PW, et al., Visualization of cell death in vivo with the annexin A5 imaging protocol, *J Immunol Methods*, 2002;265:123–32.
22. Schmitz-Valckenberg S, Guo L, et al., *In vivo* imaging of retinal cell apoptosis following acute light exposure, *Ophthalmologe*, 2010;107:22–9.
23. Schmitz-Valckenberg S, Guo L, et al., Real-time *in-vivo* imaging of retinal cell apoptosis after laser exposure, *Invest Ophthalmol Vis Sci*, 2008;49:2773–80.
24. Fitzke F, Imaging the optic nerve and ganglion cell layer, *Eye*, 2000;14:450–53.
25. Nickells RW, Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death, *Surv Ophthalmol*, 1999;43:S151–61.
26. Tatton NA, Tezel G, Insolia SA, et al., In situ detection of apoptosis in normal pressure glaucoma. a preliminary examination, *Surv Ophthalmol*, 2001;45:S268–72, discussion S273–266.
27. Weinreb RN, Friedman DS, Fechtner RD, et al., Risk assessment in the management of patients with ocular hypertension, *Am J Ophthalmol*, 2004;138:458–67.
28. Okisaka S, Murakami A, Mizukawa A, Ito J, Apoptosis in retinal ganglion cell decrease in human glaucomatous eyes, *Jpn J Ophthalmol*, 1997;41:84–8.
29. Morrison JC, Moore CG, Deppmeier LM, et al., A rat model of chronic pressure-induced optic nerve damage, *Exp Eye Res*, 1997;64:85–96.
30. Shareef S, Garcia-Valenzuela E, Salierno A, et al., Chronic ocular hypertension following episcleral venous occlusion in rats, *Exp Eye Res*, 1995;61:379–82.
31. WoldeMussie E, Ruiz G, Wijono M, Wheeler LA, Neuroprotection of retinal ganglion cells by brimonidine in rats with laser-induced chronic ocular hypertension, *Invest Ophthalmol Vis Sci*, 2001;42:2849–55.
32. Ueda J, Sawaguchi S, Hanyu T, et al., Experimental glaucoma model in the rat induced by laser trabecular photocoagulation after an intracameral injection of India ink, *Jpn J Ophthalmol*, 1998;42:337–44.
33. Levkovitch-Verbin H, Quigley HA, Martin KR, et al., Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats, *Invest Ophthalmol Vis Sci*, 2002;43:402–10.
34. Sawada A, Neufeld A, Confirmation of the rat model of chronic, moderately elevated intraocular pressure, *Exp Eye Res*, 1999;69:525–31.
35. Mittag TW, Danias J, Pohorenec G, et al., Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model, *Invest Ophthalmol Vis Sci*, 2000;41: 3451–9.
36. Naskar R, Wissing M, Thanos S, Detection of early neuron degeneration and accompanying microglial responses in the retina of a rat model of glaucoma, *Invest Ophthalmol Vis Sci*, 2002;43:2962–8.
37. Bayer AU, Danias J, Brodie S, et al., Electroretinographic abnormalities in a rat glaucoma model with chronic elevated intraocular pressure, *Exp Eye Res*, 2001;72: 667–77.
38. Agar A, Yip SS, Hill MA, Coroneo MT, Pressure related apoptosis in neuronal cell lines, *J Neurosci Res*, 2000;60:495–503.
39. Levkovitch-Verbin H, Quigley HA, Kerrigan-Baumrind LA, et al., Optic nerve transection in monkeys may result in secondary degeneration of retinal ganglion cells, *Invest Ophthalmol Vis Sci*, 2001;42:975–82.
40. Harwerth RS, Carter-Dawson L, Smith EL, 3rd, et al., Neural losses correlated with visual losses in clinical perimetry, *Invest Ophthalmol Vis Sci*, 2004;45:3152–60.
41. Quinn R, Comparing rat's to human's age: how old is my rat in people years?, *Nutrition*, 2005;21:775–7.
42. Weinreb RN, Kaufman PL, The glaucoma research community and FDA look to the future: a report from the NEI/FDA CDER Glaucoma Clinical Trial Design and Endpoints Symposium, *Invest Ophthalmol Vis Sci*, 2009;50:1497–1505.
43. Oliver JE, Hattenhauer MG, Herman D, et al., Blindness and glaucoma: a comparison of patients progressing to blindness from glaucoma with patients maintaining vision, *Am J Ophthalmol*, 2002;133:764–72.
44. Collaborative Normal-Tension Glaucoma Study Group T, Comparison of glaucomatous progression between untreated patients with normal-tension glaucoma and patients with therapeutically reduced intraocular pressures. Collaborative Normal-Tension Glaucoma Study Group, *Am J Ophthalmol*, 1998;126:487–97.
45. Osborne NN, Ugarte M, Chao M, et al., Neuroprotection in relation to retinal ischemia and relevance to glaucoma, *Surv Ophthalmol*, 1999;43:S102–28.
46. Lipton SA, Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders, *J Alzheimers Dis*, 2004;6:S61–74.
47. Farlow MR, NMDA receptor antagonists. A new therapeutic approach for Alzheimer's disease, *Geriatrics*, 2004;59:22–7.
48. Hare W, WoldeMussie E, Lai R, et al., Efficacy and safety of memantine, an NMDA-type open-channel blocker, for reduction of retinal injury associated with experimental glaucoma in rat and monkey, *Surv Ophthalmol*, 2001;45:S284–9, discussion S295–86.
49. WoldeMussie E, Yoles E, Schwartz M, et al., Neuroprotective effect of memantine in different retinal injury models in rats, *J Glaucoma*, 2002;11:474–80.
50. Schmitz-Valckenberg S, Guo L, Maass A, et al., Real-time in vivo imaging of retinal cell apoptosis after laser exposure, *Invest Ophthalmol Vis Sci*, 2008;49:2773–80.
51. Guo L, Duggan J, Cordeiro MF, Alzheimer's disease and retinal neurodegeneration, *Curr Alzh Res*, 2010;7:3–14.