Involvement of ascorbic acid in iron-induced apoptosis in the retina

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Abstract

Aim: To investigate the correlation of ascorbic acid distribution in the retina to the occurrence of iron-induced apoptosis in the retina.

Materials and methods: Apop-Tag, an *in situ* apoptosis detection kit, was used to visualize the distribution of apoptosis after intraocular iron implantation in rat and rabbit eyes. Ascorbic acid distribution was visualized by the Chinoy method.

Results: On day 10, after intraocular implantation of iron, most of the outer nuclear layer was destroyed by apoptosis. However, no apoptotic nucleus was observed in the ganglion layer and inner nuclear layer of the rats' eyes. On the contrary, apoptosis was observed in all layers in rabbits' retinas on day 3. Ascorbic acid distribution in eye tissues was visualized by the silver granules produced from the reduction of silver nitrate by ascorbic acid. In normal rats' retinas silver granules were observed only in the outer nuclear layer, while silver granules were distributed evenly in all layers of the rabbits' retinas. The distribution of ascorbic acid in the retina coincided with the occurrence of iron-induced apoptosis.

Conclusion: Iron toxicity is due to free radical formation during auto-oxidation of ferrous to ferric ions. The high amount of ascorbic acid in the outer nuclear layer of the rats' retinas regenerate ferric ion to ferrous ion allowing for continuous production of free radicals in the outer nuclear layer. This conclusion was supported by the distribution of apoptosis in all layers of the rabbits' retinas where ascorbic acid is evenly distributed in all layers of the rabbits' eyes.

Key words: *Ascorbic acid, Free radical, Iron, Apoptosis, Retina*

Introduction

Von Graefe called attention to the clinical problems of intraocular iron foreign bodies in 1860.[1] 32 years later, Bunge introduced the term 'siderosis bulbi' for the rust colored eye balls.[2] Iron deposits in the retina produce severe loss of vision known as siderotic retinal degeneration. Lens opacity induced by iron is called siderotic cataract. Iron-induced obstruction of aqueous humor drainage is known as glaucoma secondary to siderosis.[3,4] Furthermore, ocular siderosis can result from iron released from hemoglobin, now known as hemosiderosis.[5] Epidemiologic study shows that most foreign bodies in penetrating ocular injuries contain iron.[6-9] The damage to ocular structures by iron is a serious problem, especially for patients in whom the seriousness of the condition

prevents immediate surgical removal of the foreign body. Therefore, an in-depth investigation of the mechanism of intraocular iron toxicity has both medical and social significance.

Most investigations of the toxicity of intraocular iron foreign body were carried out in the 1970s. Since then, our knowledge of the biochemistry of iron toxicity and the basic pathology of the retina, as well as the mechanism of cell death has greatly increased. Apoptosis has been shown to be the major mechanism of retinal degeneration.[10] Our recent report showed that the intravitreal implantation of an iron particle induced apoptosis in the outer nuclear layer (ONL) of a rat's retina.[11] The limitation of iron toxicity to the ONL is a puzzling phenomenon. A trace amount of ferrous ion released from an iron particle in the vitreous cavity should have been converted to the stable ferric ion as it diffused toward the retina. One does not expect that a sufficient amount of ferrous ion could have reached the ONL to induce apoptosis without an effect on the ganglion layer (GL) and the inner nuclear layer (INL). The present study was carried out to explain the puzzling observation based on ascorbic acid involvement in iron-induced apoptosis.

Materials and methods

Autoclaved 5 mg iron particles (99.98%, Aldrich, Milwaukee, WI, USA) were used in this experiment. Apop-Tag, an *in situ* apoptosis detection kit, was supplied by Oncor Co. Inc. (Gaithersburg, MD, USA). Proteinase K and DNase free RNase A were supplied by Sigma (St Louis, MO, USA).[11] The foreign body was inserted into the vitreous cavity by a micro-pick monitored under microscope as previously described.[11] The conjunctival flap was closed by cauterization. Maxitrol (Alcon, No. 00630, Fort Worth, TX, USA) ointment was administrated at the end of the operation. Maxitrol eye drops were given daily after each ocular examination.

All eyes were enucleated and fixed in 10% buffered formaldehyde. The eyes were opened anterior-posteriorly and the iron particle was removed after overnight fixation. Tissue sections were examined after hematoxylin-eosin (H&E) staining and TdT-mediated dUTP-biotin nick end labeling (TUNEL) as previously described.[11]

Chinoy's silver nitrate staining method was used to visualize ascorbic acid distribution in the retina.[12] The data presented in this report has been reproduced in 20 eyes of normal Sprague Dawley rats and 16 normal New Zealand rabbits. The tissue sections were counter stained in 1% methyl green. The animals were fed *ad libitum* and maintained with a 12-hour light/dark cycle.

Results

Effect of intraocular iron on rats' retinas examined by H&E staining

The rapid cell loss in the rats' ONL beginning on day 2 after iron implantation was clearly seen in H&E stained specimens (**Figures 1 to 5**). **Figure 1** shows a normal Sprague Dawley rat retina with a well-organized structure. GL, INL, and ONL are shown from top to bottom. There were no noticeable changes before day 2 after intraocular iron implantation. Progressive cell loss in the ONL began on day 2 (**Figure 2**) and rapidly spread throughout the ONL by day 3. Severe damage to the ONL was already noticeable on day 2.5 (**Figure 3**). Most of the ONL was lost by the end of day 3 (**Figure 4**). On day 7, (**Figure 5**) only a few cells remained in the ONL and massive infiltration of macrophages appeared. When a piece of glass was

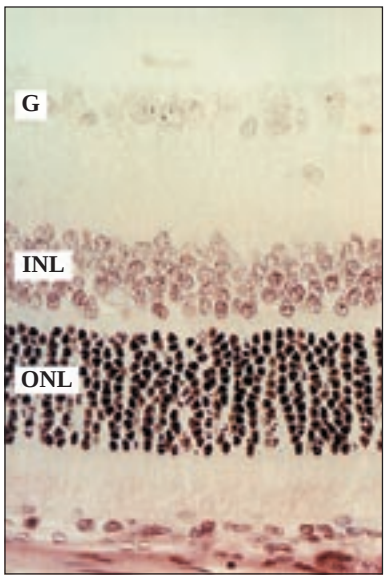

Figure 1. Well-organized normal rat retina. H&E staining (x400).

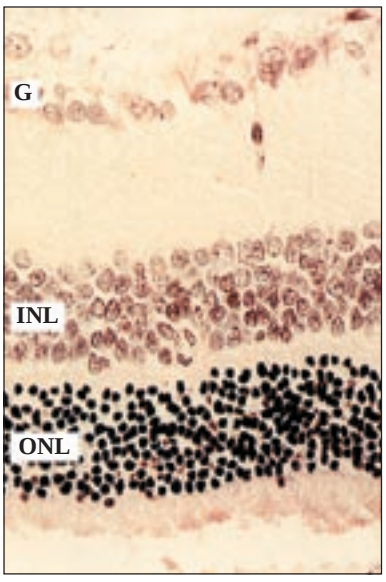

Figure 2. Rat's retina on day 2; slightly disorganized and condensed nuclei appeared in the outer nuclear layer. H&E staining (x400).

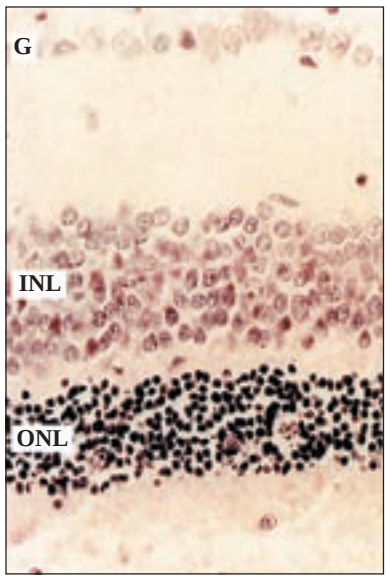

Figure 3. Rat's retina on day 2.5; moderate cell loss in the outer nuclear layer. H&E staining (x400).

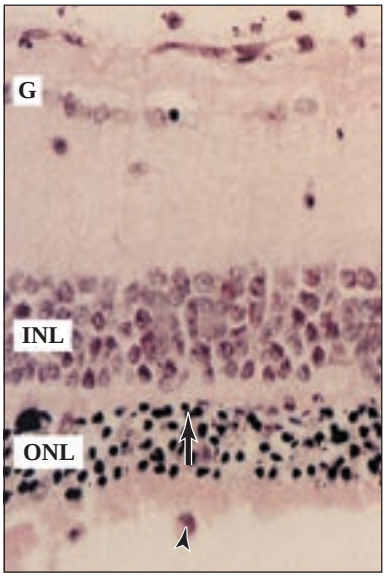

Figure 4. Rat's retina on day 3; large cell loss in the outer nuclear layer. H&E staining (x400).

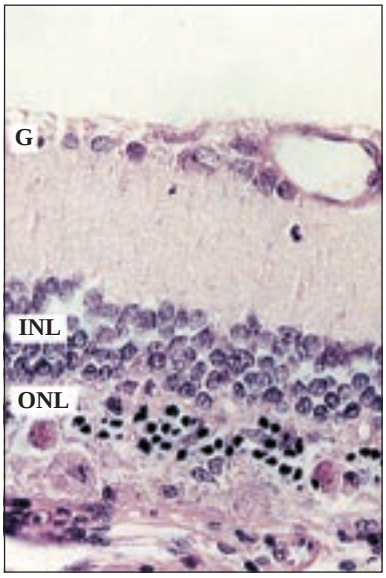

Figure 5. Rat's retina on day 7; very few cells remain in the outer nuclear layer. H&E staining (x400).

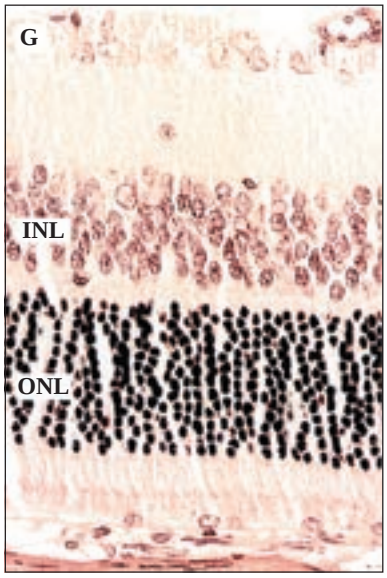

Figure 6. A control rat's retina on day 7 after intraocular implantation of a glass chip. Negligible change in the retina was induced by the glass chip. H&E staining (x400).

implanted into the vitreous cavity of a control rat, there was a negligible effect in the retina up to day 7 (**Figure 6**).

Effect of intraocular iron in rats' retinas examined after staining for apoptosis

The magnified pictures of the ONL illustrate the rapid rise in the severity of apoptosis immediately after day 2 (**Figures 7 to 10**). On day 2, a few cells with a brownish stain around their nuclei indicated the beginning of activated endonuclease (**Figure 7**). A marked increase in the number of apoptotic nuclei and brownish staining of disintegrated cells were noticeable on day 2.5 (**Figure 8**). The label-positive substance intensified markedly by day 3 (**Figure 9**). Macrophages were noticed in the ONL as early as day 3. Two macrophages with phagocytized TUNEL-positive nuclei in the ONL (day 7) are shown in **Figure 10**.

Effect of intraocular iron on rabbits' retinas

The effect of intraocular iron in rabbits' eyes was quite different from that in rats' eyes. The specimen taken on day 3 was chosen to illustrate the occurrence of apoptosis in all

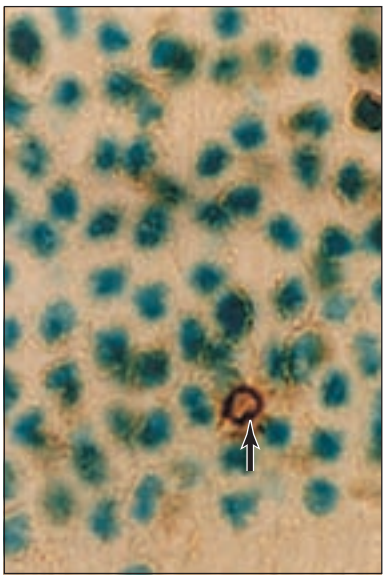

Figure 7. Rat's retina on day 2; a typical label-positive cell (arrow). TUNEL (x1300).

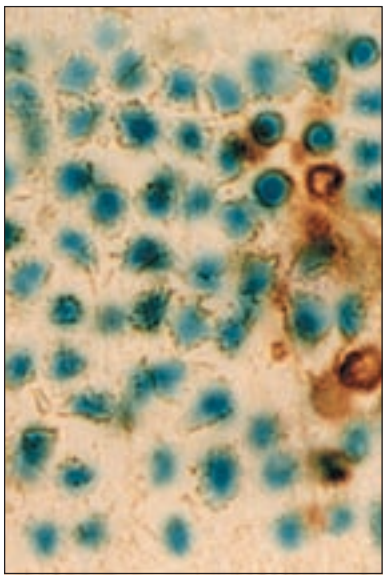

Figure 8. Rat's retina on day 2.5; increased number of apoptotic nuclei. TUNEL (x1300).

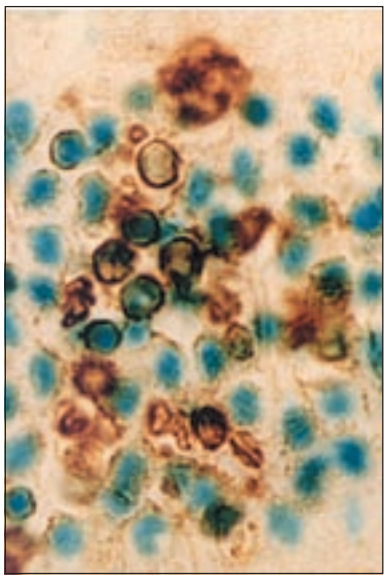

Figure 9. Rat's retina on day 3; markedly increased number of apoptotic nuclei and damaged cells. TUNEL (x1300).

retinal layers of rabbits in the early stage of intraocular iron toxicity. The H&E staining showed nuclear condensation in the GL, INL, and ONL (**Figure 11**). The occurrence of apoptosis in all cell layers is clearly illustrated in **Figure 12** by the TUNEL technique. Despite total damage to the ONL of the rat's retina on day 10, there were no apoptotic cells noticeable in the GL and INL (**Figure 13**).

The distribution of ascorbic acid in the retinas of rats and rabbits

Chinoy's method was used to compare ascorbic acid distribution between rats' and rabbits' retinas.[12] Ascorbic acid in the tissue reduced silver nitrate to black silver granules. When a normal rat retina was examined, silver granules were observed mainly in the ONL and choroid (**Figure 14**). When a rabbit retina was examined, silver granules were observed evenly all over the retina (**Figure 15**).

Discussion

Our previous report described a rapid progression of apoptosis within the ONL of rats' retinas beginning on day 2 after intraocular iron implantation.[11] We have now extended the previous observation to 10 days. Despite total destruction of the ONL on day 10, there were no noticeable TUNEL-positive cells in the GL and INL (**Figure 13**). Macrophages with phagocytized TUNEL-positive nuclei were also confined to the area of the ONL (**Figure 10**). The present data clearly documents the limitation of iron-induced apoptosis within the rats' ONL

The distribution of ascorbic acid in the rats' retinas provides an explanation for the limitation of apoptosis in the ONL of rats' retinas. There was negligible ascorbic acid in the vitreous humor of rats.[13] Iron in the vitreous humor should have existed as the stable ferric ion. As a trace amount of the inert ferric ion diffused into the rats' ONL, it was reduced by ascorbic acid to ferrous ion. The auto-oxidation of a ferrous ion to ferric ion released a single electron to form the highly toxic superoxide. A continuous turnover of ferric-ferrous ions by a high concentration of ascorbic acid continuously generated free radicals in the ONL. Free radicals are very unstable. Their toxicity is limited to the location where they are being generated. Therefore, the toxicity of free radicals produced from ascorbic acid driven oxidation-reduction of iron was confined within the rats' ONL.

The above interpretation was verified by comparing the correlation of apoptosis and ascorbic acid distribution in rats' and rabbits' eyes. Ascorbic acid concentration was very high in rabbits' vitreous humor and evenly distributed throughout the rabbits' retinas **(figure 15)**.[14] Therefore, iron should have been reduced to ferrous ion by ascorbic acid in the vitreous cavity. As ferrous ions diffused into the retina, they initiated apoptosis in all retinal layers in the rabbits' eyes (**Figure 12**). The rabbits' eyes (**Figure 12**) were enucleated on day 3 after iron implantation. Day 3 was chosen in order to show that apoptosis occurs in all retinal layers of rabbits' eyes at an early stage of iron toxicity contrary to the limitation of apoptosis in the ONL of rats' retinas up to the late stage of iron toxicity (**Figure 13**).

This interpretation was based on the assumption that the location of silver granules resulted from the reduction of silver nitrate by ascorbic acid. The fixative and staining method was designed by Chinoy to eliminate interaction between silver nitrate to the major physiological reducing

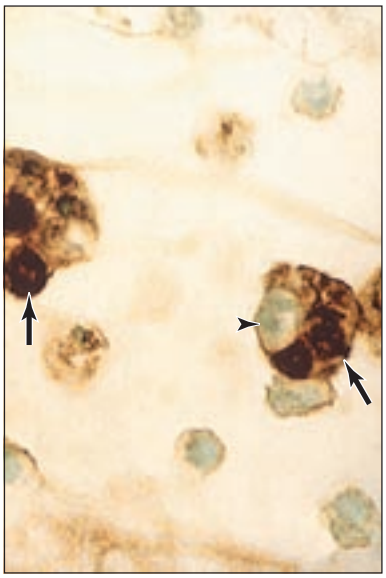

Figure 10. Rat's retina on day 10; two macrophages (arrow) with phagocytized TUNEL-positive nuclei. The nucleus of the macrophage was stained green by counter stain (arrowhead). TUNEL (x1300).

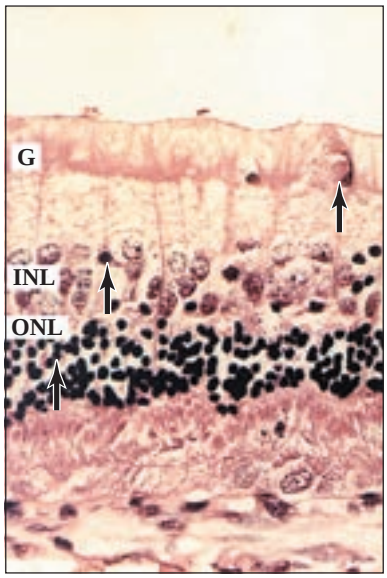

Figure 11. Rabbit retina on day 3; nuclear condensation was noticed in the granular layer, inner nuclear layer, and outer nuclear layer. H&E staining (x400).

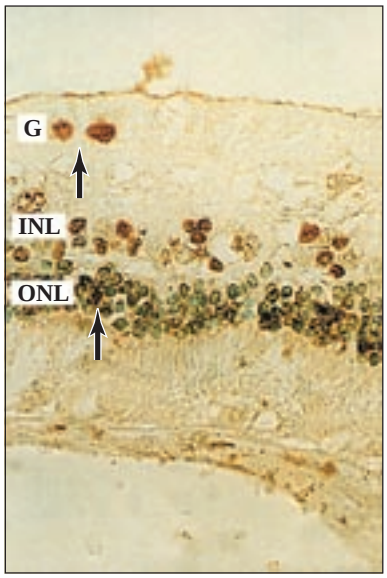

Figure 12. Rabbit retina on day 3; TUNEL-positive nuclei appeared in the granular layer, inner nuclear layer, and outer nuclear layer. TUNEL (x400).

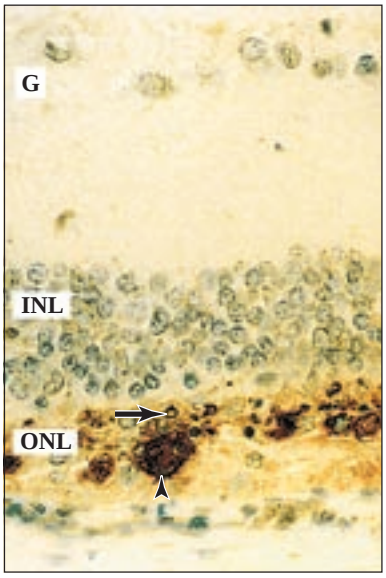

Figure 13. Rat retina on day 10; TUNEL-positive nuclei appeared only in the outer nuclear layer. TUNEL (x400).

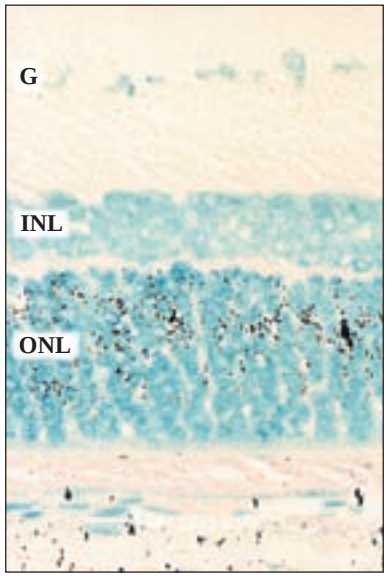

Figure 14. Normal rat's retina; dense silver granules were observed in the outer nuclear layer. Silver nitrate staining.

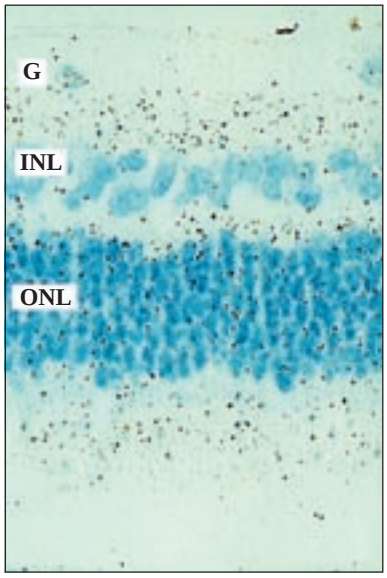

Figure 15. Normal rabbit's retina; black silver granules were observed in all retinal layers. Silver nitrate staining.

agents such as glutathione, sugars, and amino acids.[12] Introduction of iron into the vitreous cavity induced oxidation of endogenous ascorbic acid and abolished the appearance of silver granules. The difference observed between the distribution of silver granules in the eyes of rabbits and rats is consistent with previous chromatographic analysis of ascorbic acid concentrations in ocular tissues and fluid. Previous reports have demonstrated that rabbits have a very high ascorbic acid concentration in the vitreous humor and throughout all eye tissues,[14] while ascorbic acid in rats is limited to the retina.[13] The silver staining method allows us to visualize the distribution of ascorbic acid among different retinal layers, which cannot be achieved by chromatographic methods.

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