Iron-induced Cytotoxicity in Cultured Rat Retinal Neurons

Joonhong Sohn, M.D. and Young Hee Yoon, M.D.

Department of Ophthalmology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

Oxidative stress has been proposed as a major injury mechanism in the central nervous system including the retina. In this study, as an initial attempt to study the mechanism of oxidative injury in the retina, we developed a cell culture model by utilizing the iron exposure paradigm. Exposure of rat retinal cultures for 24 hours to 10-40 µM ferrous or ferric chloride induced a concentration-dependent death of retinal neurons but not of photoreceptors or astrocytes. An antioxidant, trolox effectively attenuated the iron-induced death of neurons and photoreceptors in a dose-dependent manner whereas neither glutamate receptor antagonists nor cycloheximide were protective. Of retinal interneurons, GABAergic neurons were more vulnerable to the iron toxicity than calbindin (+) horizontal neurons. These findings show that iron exposure induces anti-oxidant-sensitive neuronal injury in retinal culture, independent of the excitotoxic or the apoptotic mechanisms. Of retinal neurons, different cell types exhibit differential vulnerabilities to the iron-induced oxidative injury. This simplified culture model system may be useful in elucidating mechanisms of oxidative injury in the retina.

Key words: excitotoxicity, free radical, iron-induced toxicity, oxidative stress, retinal cell culture

INTRODUCTION

Oxidative stress and excitotoxicity are two main mechanisms of pathologic neuronal death in the central nervous system including the retina.\(^1\)\(^-\)\(^4\) Evidence suggests that these two pathologic processes may be closely interwoven, since 1) increases in calcium influx in excitotoxic injury promote free radical production via activation of calcium-activated phospholipases and/or nitric oxide synthase, and 2) conversely, free radicals may enhance glutamate release to potentiate excitotoxic injury.\(^5\)\(^-\)\(^6\) For this reason, often, both glutamate antagonists and anti-oxidants are effective in curtailling ischemic brain or retinal injuries.\(^3\)\(^-\)\(^7\)\(^-\)\(^9\) Although several in vitro models of excitotoxicity in the retina have been developed, relatively few exist for the study of direct oxidative injury. Clinically, the oxidative injury is implicated in various ocular diseases including ischemic or hypoxic retinal damage in retinal vascular obstruction or diabetic retinopathy, retinopathy of prematurity, cataract, age related macular degeneration, and retinal phototoxicity.\(^10\)\(^,\)\(^11\) Considering the increasing evidence for the oxidative injury in the retinal diseases, a reliable in vitro model may prove to be useful in elucidating its mechanism.

Free radicals are products of the normal energy metabolism, and hence constantly being produced in cells during oxidation-reduction reactions. In

Reprint requests to Young Hee Yoon, M.D., Department of Ophthalmology, Asan Medical Center, Ulsan University College of Medicine, 388-1 Poongnap-dong, Songpa-ku, Seoul 138-040, Korea.
excess, however, highly reactive oxygen species attack the unsaturated bonds of fatty acids and cholesterol, triggering a chain reaction that propagates to result in the formation of toxic lipid peroxides across biologic membranes.\textsuperscript{12,13} Since the retina has high levels of long chain polyunsaturated fatty acids and uses prodigious amount of oxygen, it is particularly at high risk for this type of oxidative injury. To experimentally induce lipid peroxidation and cell injury, iron has been often used, since either ferrous or ferric iron, produces cyto-destructive hydroxyl radicals via the Fenton and the Haber-Weiss reactions.\textsuperscript{14-16} The iron-induced retinal cell injury may be directly relevant in ocular siderosis, where the deposition of intraocular iron exerts severe toxic actions on retinal cells. Hence, the iron-induced injury in retinal culture may be viewed as a simplified model system for oxidative retinal injury in general, and ocular siderosis in particular. In the present study, we have sought to examine in rat primary retinal cultures, whether iron-induced retinal cell death indeed occurs mainly by oxidative stress, and whether there exist differences in vulnerability to iron toxicity among different cell types.

**MATERIALS AND METHODS**

**Retinal cell cultures**

Mixed retinal cell cultures containing both neuronal and glial elements were prepared from newborn Sprague-Dawley rats (1-2 days old). After decapitation, the retina was carefully dissected from the eye in Ca\textsuperscript{2+} and Mg\textsuperscript{2+} free Eagle’s minimum essential medium (MEM, GIBCO) and then dissociated into single cells by trituration using fire-polished Pasteur pipettes. The dissociated retinal cells were plated on MEM supplemented with 10% horse serum, 10% bovine serum, and 38 mM KCl and then, plated on poly-L-lysine coated 15 mm 24-multitwell plates (Nunc) at the density of 3 retinas per plate. Cultures were kept at 37°C in an incubator containing 5% carbon dioxide. The cultures between 7 to 10 days in vitro were used for experiments.

**Exposure to drugs**

After a triple-wash of the cell cultures with MEM supplemented with 38 mM KCl, ferrous or ferric chloride was added to the media at various concentrations between 10 and 40 μM. The cultures were then placed back into the incubator until the next day when the evaluations were made. To test the effect of potential cytoprotective agents, chemicals were added along with iron throughout the exposure duration (24 h). A vitamin E analog, trolox, was added at concentrations of 100 or 400 μM, the N-methyl-D-aspartate (NMDA) antagonist dizocilpine (MK-801), at 10 μM, the alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA)/kainate receptor antagonist, 6-nitro-7-cyanoquinoxaline-2,3-dion (CNQX), at 50 μM, and the protein synthesis inhibitor cycloheximide, at 1 μg/ml.

**Immunocytochemical staining**

Cultures were fixed by adding 1% glutaraldehyde in potassium phosphate buffered saline (PBS, pH7.4) for one hour at room temperature and then washed with excess PBS to remove all the fixative. The fixed cultures were incubated for 20 min in PBS containing the specific antibody to mouse microtubule associated protein (MAP)-2 (1/500, Sigma), or rabbit anti-mouse gamma aminobutyric acid (GABA) (1/1000, Sigma), or mouse anti-rat calbindin D-28K (1/500, Sigma) in the presence of 0.1% triton X-100. After thorough rinsing, cultures were incubated in PBS containing the respective biotin-labeled secondary antibody for 1 h at room temperature. Secondary antibodies were; anti-mouse IgG for anti-MAP 2 and anti-calbindin D 28K antibody, and anti-rabbit IgG for anti-GABA antibody. Finally, cultures were incubated with the peroxidase-avidin complex for 30 minutes at room temperature, and then the conjugated complex was visualized by the reaction with the chromogen, 3-amino-9-ethylcarbazole.

**Assessment of cellular injury**

As reported previously,\textsuperscript{8,17-20} the retinal cultures contained three types of cellular components. Neuronal cells having polygonal cell bodies and several long slender processes were stained with anti-MAP 2 antibody. Astroglial cells that form a background monolayer were stained with anti-GFAP antibody. Cells with small round cell bodies without processes were classified as photoreceptors. These cells often formed rosettes.
The overall neuronal cell injury was estimated by observing the cells under a phase contrast microscope (Olympus, Japan). Morphological assessment was confirmed by the trypan blue staining technique. For quantitative assessment, the viable cells were counted in five 200X microscopic fields in two culture wells with the following an immunocytochemical stainings: anti-MAP 2 antibody staining for all neuronal cells, anti-GABA antibody staining for GABAergic interneurons, and anti-calbindin antibody staining for calbindin-containing horizontal cells. Each field for counting was selected randomly. For statistical comparison, the two tailed test was used.

Terminal deoxynucleotidyl transferase-mediated nick labeling (TUNEL) stain

TUNEL staining technique was used for the in situ visualization of DNA breaks. Cultures were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature and then washed with PBS. Fixed cultures were treated with 0.3% H2O2 in methanol for 30 minutes and treated with 0.1% Triton X-100 in PBS for 5 minutes. The cultures were treated with 50 μM of TUNEL reaction mixture and then kept in the incubator at 37°C for 60 minutes. Then the cultures were washed with PBS profusely and treated with the signal OD. Finally diaminobenzidine was added for fluorescent labeling.

RESULTS

Exposure of retinal cultures for 24 h to ferrous (Fe^{2+}) chloride induced gradually developing cell injury, accompanied by cell body swelling, in retinal cultures. The extent of cell death in neurons as a whole, was quantitatively assessed by counting surviving cells that were stained with a neuron-specific anti-MAP2 antibody. The number of surviving neurons decreased in Fe^{2+} concentration-dependent manner (Fig. 1). At 40 μM concentrations, iron killed almost all the MAP-2 (+) neurons. Ferric (Fe^{3+}) iron induced similar pattern of neuronal death in the same concentration range (not shown).

Compared to MAP2 (+) neurons, the photoreceptors were less vulnerable to iron exposure, since they maintained normal morphology and excluded trypan blue after the iron exposure (not shown). Interestingly, even among retinal neurons, differences in vulnerability to iron toxicity were observed. The calbindin (+) horizontal cells around the photoreceptor rosettes were highly resistant to iron toxicity (Fig. 2). By contrast, GABA (+) interneurons, presumably amacrine and bipolar cells, were highly vulnerable; with 40 μM iron exposure, almost complete loss of GABA (+) neurons were noted (Fig. 3).

Although many studies report interconnection between excitotoxicity and oxidative injury, in the present retinal cultures, addition of glutamate receptor antagonists (MK-801 or CNQX) failed to attenuate iron induced neuronal damage, indicating that excitotoxic mechanism may not be involved in this case. Furthermore, a protein synthesis inhibitor, cycloheximide did not attenuate neuronal death, indicating that the death may not be protein synthesis-dependent apoptosis, either. However, this iron-induced damage was completely blocked by the addition of an antioxidant trolox (Fig. 4).

However, the TUNEL method revealed that the degenerating neurons after 24 h exposure to Fe^{2+} developed in situ DNA breaks (Fig. 5). Interestingly, photoreceptor cells (arrow) did not exhibit any DNA breaks, although they were surrounded by TUNEL (+) neurons. Despite DNA breaks, nuclear fragmentation, a hallmark feature of classical apoptosis, was absent.

DISCUSSION

Similar to the results in cortical neurons, adding iron (Fe^{2+} or Fe^{3+}) to retinal culture induced antioxidant-sensitive neuronal death in iron concentration-dependent fashion. But different from some studies in cortical neuronal cultures, glutamate receptor antagonists did not prevent the iron-induced neuronal injury in retinal culture. This finding alone, however, does not rule out the possibility that oxidative and excitotoxic injuries sometimes present in a closely inter-linked form in the present retinal culture. For example, in another model of oxidative injury induced by ultraviolet exposure, the cellular damage was significantly attenuated by the addition of glutamate receptor antagonists (unpublished). In addition, although the TUNEL method
stained injured neurons, this may not be sufficient evidence to call the death apoptosis, since the method is clearly non-specific, especially when it was not accompanied by the nuclear fragmentation, presumably a sine qua non of classical apoptosis. Furthermore, since the death was accompanied by cell body swelling and not attenuated by cycloheximide, apoptosis may not be the main mechanism of iron-induced neuronal death. Hence, depending on the types of oxidative insults, contribution from excitotoxicity or apoptosis may differ in the extent. Since other injury mechanisms may not be importantly involved, the present model of iron toxicity may be regarded as a relatively pure oxidative injury model compared with those in other cultures.

Our results additionally demonstrate differential vulnerability of various retinal cell types to iron-induced injury. As in cortical cultures, following iron exposure, neuronal cells were preferentially damaged compared with glial cells in the retinal culture. In addition, photoreceptor cells exhibit remarkable resistance to iron toxicity. Of neuronal types, GABAergic interneurons, mostly amacrine cells and some bipolar cells, were much more vulnerable to
the iron toxicity than calbindin (+) horizontal cells. Interestingly, GABAergic neurons in retinal culture also exhibit increased vulnerability to hypoxic injury, where oxidative stress may play a key role.\textsuperscript{8} In addition, these neurons have been shown to be preferentially damaged by AMPA/kainate receptor-mediated excitotoxicity. Since these interneurons in vivo are also highly vulnerable to hypoxia-ischemia\textsuperscript{2}, it is tempting to speculate that the oxidative stress, in addition to AMPA/kainate receptor-mediated excitotoxicity, may be the key contributing injury mechanism therein.

One of the interesting observations that we made was that horizontal cells, which usually gathered around the photoreceptor rosette, showed strong resistance to the iron induced damage. Since the horizontal cells contain calbindin, a calcium binding protein, one possible explanation may be that calbindin may bind iron to prevent its toxic action, or alternatively, may bind calcium to mitigate the secondary calcium-mediated cytotoxicity.

Clinically, the decrease in the b-wave amplitude is often the first sign in both ocular ischemia and ocular siderosis.\textsuperscript{22,23} These electroretinographic findings may functionally represent the particular susceptibility of amacrine or bipolar cells to the free radical induced toxicity, as compared with photoreceptors, consistent with the selective vulnerability of GABA (+) neurons, compared with photoreceptors and horizontal neurons, as demonstrated in the pre-
Fig. 4. Pharmacology of iron toxicity to MAP-2 (+) retinal neurons. Photomicrographs of sister retinal cultures after 24 h exposure to 20 µM Fe²⁺ alone (A), or with addition of 10 µM MK-801 (B), 50 µM CNQX (C), 1 mg/ml cycloheximide (D), or 100 (E) or 400 µM trolox (F). Only the antioxidant trolox attenuated the neuronal loss induced by iron exposure. G) Bars denote number of surviving neurons per 200X field, after 24 h exposure to 20 (filled) or 40 µM (shaded) Fe²⁺ alone or with addition of indicated drugs. Asterisks denote difference from respective Fe alone (p < 0.05, two tail t test).
Fig. 5. In situ DNA breaks occur in neurons after exposure to Fe^{2+}. Photomicrographs of sister retinal cultures with sham wash (A) or after 24 h exposure to 40 mM Fe^{2+} (B at 100X, and C at 200X). Despite the widespread emergence of TUNEL (+) neurons (arrow), photoreceptors in the center were completely devoid of TUNEL (+) cells. Of note, however, no nuclear fragmentation, a typical feature of classical apoptosis, was seen.

sent study. Although intravitreal injection of iron injured photoreceptors in addition to other retinal neurons in squirrel monkeys (Masciulli et al.), it has not looked at the low end of iron exposure to search for the possible occurrence of selective neuronal injury, hence leaving the possibility still feasible. Alternatively, the discrepancy may reflect the yet unidentified, genuine cell biological differences between in vitro and in vivo photoreceptors.

In summary, iron-exposure in primary retinal cultures, may be useful for the study of the mechanism of oxidative injury in general, and ocular siderosis-associated injury in particular. Further studies on the molecular basis for differential vulnerability of different cell types to oxidative injury may help provide insights into the selective pathogenic processes in the retina in various conditions.

REFERENCES


