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FREE RADICAL ENHANCER XENOBIOTIC IS AN INDUCER OF CATARACT IN RABBIT

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Free radical enhancers, diquat, paraquat, plumbagin and juglone were used to study the oxy radical-induced damage to the rabbit lens *in vitro* and *in vivo*. Each compound caused a 6–8 fold increase in malondialdehyde (MDA) and a 30–55% decrease in reduced glutathione of the lens *in vitro*. These peroxidative and oxidative changes were potentiated in the presence of 100% O_2, abolished by N_2 and prevented by desferal-Mn (III) (DF-Mn) or liposomal superoxide dismutase (LSOD) indicating the involvement of $O_2^{\cdot -}$.

Diquat injected intravitreally as a single dose (300 nmole in 30 μl of isotonic saline) in the right eye of a 5-wk-old Dutch belted rabbit, induced early cataract after 24–72 h. The lens of the contralateral control eye injected with isotonic saline had no change. In the right eye, $O_2^{\cdot -}$ and OH· productions were significantly ($P < 0.01$) higher; $O_2^{\cdot -}$ was about 16 fold higher in the aqueous humor and vitreous humor, and 5 fold in the lens and retina, and OH· was 35 fold higher in the aqueous humor, 2 fold in vitreous humor and 5 fold in the lens and retina as compared to the respective tissues of the control eye. Enhanced lipid peroxidation in the lens was apparent from the higher levels of MDA and formation of aminophospholipid·MDA Schiff-base conjugates.

We propose that cyclic oxidation-reduction of xenobiotics coupled to the endogenous redox systems in the eye, could generate oxy radicals in excessive amounts, triggering cataractogenesis.

KEY WORDS: Xenobiotics, diquat, oxygen free radicals, desferal-Mn(III), liposomal SOD, cataract.

INTRODUCTION

Bipyridylium compounds, 1,1′-Ethylene-2,2′-bipyridylium dibromide (Diquat) and 1,1′-dimethyl-4,4′-bipyridylium dichloride (Paraquat), and compounds having quinonoid moieties such as 5-hydroxy-1,4-naphthoquinone (Juglone), 5-hydroxy-2-methyl-1,4-naphthoquinone (Plumbagin) are univalently reduced within cells to their free radical forms which under aerobic condition, react spontaneously with the molecular oxygen generating superoxide anion free radical[1-4] (Figure 1). The cellular toxicity of these xenobiotics has been shown to be mediated by the free radicals produced during their reduction and oxidation.[1-4] Reduction of diquat and paraquat to free radicals, and their subsequent autoxidation producing H_2O_2, has been considered as the mechanism of their herbicidal action.[5] The cataractogenic effect of diquat in rats and dogs has been previously reported.[6,7] In an *in vitro* study,[8] it has been shown that $O_2 \cdot^-$ is produced during aerobic oxidation of diquat free radical. The formation of diquat free radical by a reaction catalyzed by glutathione reductase in the bovine lens extract in the presence of NADPH, and the production of H_2O_2 by diquat catalyzed aerobic oxidation of ascorbic acid in bovine aqueous humor and vitreous humor exposed to sunlight *in vitro*, have also been demonstrated earlier.[9] However, there is no evidence

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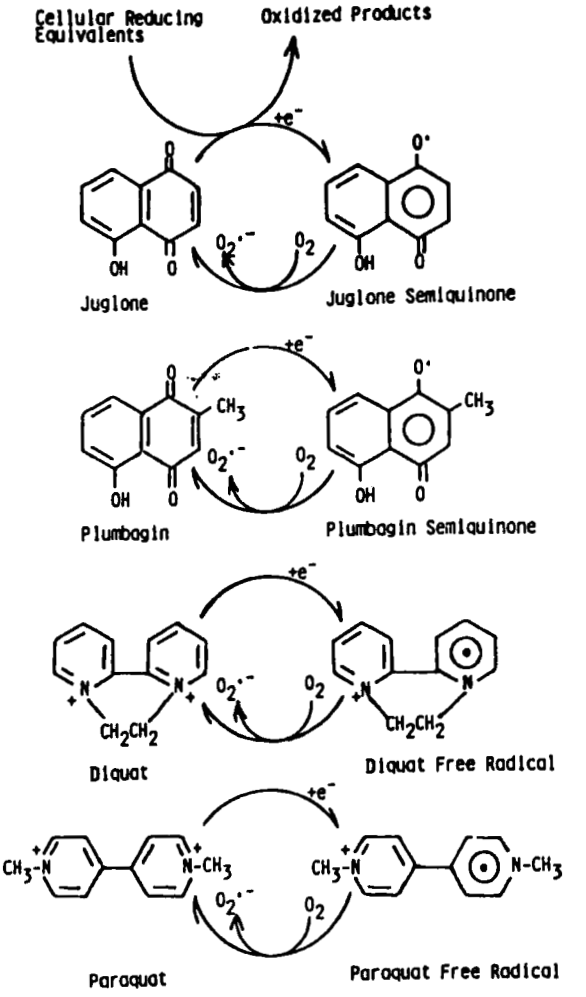

FIGURE 1 A schematic representation of the reactions showing a single electron transfer from the possible endogenous cellular reductant, such as ascorbic acid, GSH, NADPH, glutathione reductase, reduced flavins, or microsomal reductases, to the redox active xenobiotic, reducing it to its free radical intermediate form which further donates an electron to the molecular oxygen liberating superoxide free radical and the parent compound.

in the literature to suggest the notion that diquat-induced cataract is mediated by oxy radicals.

In this communication we describe results to show that oxidative damage to the lens induced by redox xenobiotics *in vitro* and production of cataract in rabbit by diquat *in vivo*, are triggered by oxy radicals.

MATERIALS AND METHODS

Chemicals

Diquat dibromide monohydrate (100 % pure), was a gift from the Imperial Chemical

Industries plc.), London, U.K., paraquat dichloride (approx. 98 %), Juglone (98 %), plumbagin (approx. 98 %), disodium ethylenediaminetetraacetate (EDTA), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2,6-dichlorophenolindophenol sodium salt (Grade I), cytochrome *c* (Type IV) from horse heart, superoxide dismutase (SOD, purified from bovine erythrocyte, 3250 units/mg protein), and all other chemicals of analytical grade were purchased from the Sigma Chemical Company. The inorganic chemicals of certified grade were from the Fisher Scientific Company. Desferal-Mn(III) (DF-Mn) was prepared according to the procedure of Fridovich and associates.[10] Liposomal SOD (LSOD) was a gift from Professor A. Michael Michaelson from the Institute de Biologie Physico-chimique, Paris.

Test Animals

The animal investigations were conducted following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Mount Sinai Medical Center, New York, and according to the resolution of the Association for Research in Vision and Ophthalmology, Inc., on the Use of Animals in Research. Healthy Dutch belted rabbits of either sex, 5- to 6-week-old, weighing 0.5 to 0.6 kg were used for the study. The animals with normal eyes were selected after examination of their eyes with a slit-lamp biomicroscope.

Lens Incubation System

Normal lenses of healthy Dutch belted rabbits weighing 1–1.5 kg were taken for *in vitro* studies. Short-term lens culture was done in a modified Krebs-Ringer bicarbonate (KRB) glucose (5 mM) medium[11] with 95 % O_2 and 5 % CO_2 as gas phase and in Krebs-Ringer (KR) HEPES (30 mM, pH 7.4) glucose medium with 100 % O_2 or N_2 as the gas phase.

Induction of Cataract

Under general anesthesia and sterile conditions, a single dose of 300 nmoles diquat dibromide in 30 μl of 145 mM NaCl was injected intravitreally to the right eye of a rabbit. The left eye was kept as control by intravitreally injecting 30 μl of the vehicle. The eyes were examined daily with a slit-lamp biomicroscope to observe the lens changes. Photographic records of the animals were kept with the help of a Zeiss photo-slit lamp biomicroscope. The eye tissues of rabbits having early cataracts, were used for the biochemical studies.

Estimation of $O_2^{\cdot-}$

In the eye tissues, $O_2 \cdot^-$ was estimated by the technique described by Fridovich,[12] which is based on the measurement of the SOD-inhibitable reduction of ferricytochrome *c* by the tissue extract at 550 nm. The assay system consisted of 0.025 mM ferricytochrome *c*, 50 mM $KH_2PO_4 \cdot K_2HPO_4$ buffer, pH 7.8, 0.1 mM EDTA, with or without 65 units of SOD and an appropriate aliquot of aqueous humor, vitreous humor or homogenate of lens or retina in a final volume of 1.3 ml incubated at 25 °C for 15 minutes. At $\varepsilon_{550\,nm}$ ferricytochrome $c = 0.89 \times 10^4 \times M^{-1} \times cm^{-1}$ and $\varepsilon_{550\,nm}$

ferrocytochrome $c = 2.99 \times 10^4 \times M^{-1} \times cm^{-1}$. The concentration of $O_2^{\cdot-}$ was calculated from the SOD-inhibitable cytochrome c reduced using $\Delta\varepsilon_{550\,nm} = 2.1 \times 10^4 \times M^{-1} \times cm^{-1}$.

Estimation of OH·

The technique described by Halliwell and Gutteridge,[13] was used to estimate OH· in eye tissues. It is based on the measurement of 2,3-dihydroxybenzoate formed by hydroxylation of salicylate by OH·. The reaction mixture consisted of 2 mM sodium salicylate, 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA and a suitable aliquot of aqueous humor or vitreous humor in a final volume of 1 ml or one preweighed lens or the whole retina from one eye in a final volume of 1.5 ml. The assay systems containing aqueous humor or vitreous humor were mixed, and those with the lens or retina were homogenized at 0–4 °C, and incubated at 25 °C for 30 minutes. The reactions were stopped by adding 40 μl of 10 M HCl and 0.25 g of NaCl/ml; 2,3-dihydroxybenzoate was extracted with chilled diethyl ether, and processed for colorimetric measurement at 510 nm. Pure 2,3-dihydroxybenzoate was used as a standard.

Estimation of H_2O_2

H_2O_2 in aqueous humor and vitreous humor was estimated by the procedure described previously.[14–16] At 610 nm, pH 6.6, reduction of 2,6-dichlorophenolindophenol (oxidized blue form) by the ascorbic acid present in a suitable aliquot of the sample was followed by measurement of the reoxidation of the reduced (leuco form) dye by H_2O_2 in the sample in presence of horseradish peroxidase. From the equimolar stoichiometry of the reactions involved, H_2O_2 was determined using $\varepsilon_{610\,nm}$ oxidized dye $= 2.1 \times 10^4 \times M^{-1} \times cm^{-1}$.

Estimation of Malondialdehyde (MDA)

MDA in the lens was analyzed by reacting the protein-free TCA supernatant with TBA.[11,17] The pink trimethine condensation product of one mole MDA with two moles TBA was measured. $\varepsilon_{533\,nm}$ MDA $= 1.5 \times 10^5 \times M^{-1} \times cm^{-1}$.

Estimation of Reduced Glutathione (GSH)

GSH in the lens was measured by reacting the protein free TCA supernatant with DTNB forming 1 mole 2-nitro-5-mercaptobenzoic acid anion per mole of -SH group.[14,18] $\varepsilon_{412\,nm}$ -SH $= 1.36 \times 10^4 \times M^{-1} \times cm^{-1}$.

RESULTS

Superoxide as the Mediator of Damaging Effects of Redox Compounds on the Rabbit Lens in vitro

The effects of a variety of compounds on lipid peroxidation (Figure 2A) and oxidation of GSH (Figure 2B) of lens in presence and absence of DF-Mn or LSOD were investigated and the results are presented graphically. It is seen from Figure 2A that

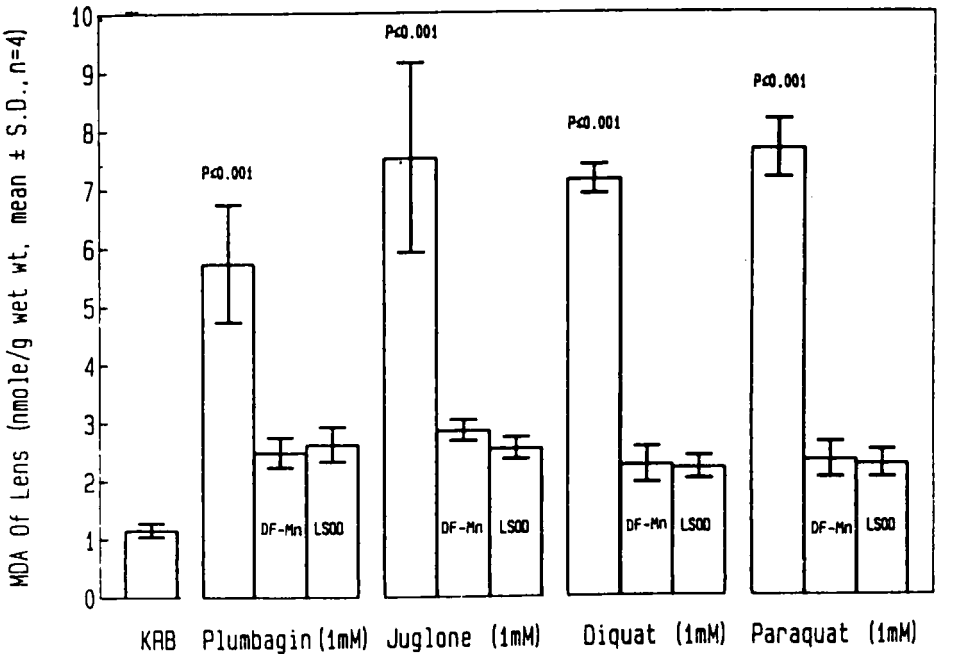

FIGURE 2A The effects of desferal-Mn(III) (DF-Mn) and liposomal superoxide dismutase (LSOD) on lipid peroxidation of the rabbit lens, induced by redox active compounds *in vitro*. Each lens was placed in a vial containing 8 ml KRB glucose medium, pH 7.4, gas phase 95 % O_2 and 5 % CO_2 with or without the addition of 1 mM plumbagin, juglone, diquat or paraquat in presence or absence of 5 mM DF-Mn or 13 μg LSOD per ml medium as indicated in the Figure. Incubation was done at 37 °C for 3 h in a water bath shaking at 72 oscillations per minute. At the end of incubation the lens was removed, rinsed with isotonic saline containing 22.6 μM BHT, and analyzed for MDA as described under "Materials and Methods" in the text.

in the normal lens incubated in KRB glucose medium for 3 h, MDA was 1.16 nmoles/g wet wt and was significantly ($p < 0.001$) higher, 5 fold with 1 mM plumbagin and about 6–7 fold with 1 mM juglone, paraquat or diquat as compared to control values. Addition of 5 mM DF-Mn or 13 μg LSOD/ml to the incubation systems produced significantly ($P < 0.001$) lower values of the augmented peroxidative damage to the lens in presence of redox compounds to about 2 fold. Figure 2B shows that GSH in the normal lens at the end of 3 h incubation in KRB glucose medium, was 8.74 μmoles/g wet wt. As compared to this value, the lens GSH was significantly ($P < 0.01$) lower by 30 % in the presence of paraquat and about 50 % in the presence of diquat, plumbagin or juglone. Addition of DF-Mn or LSOD to the incubation system containing the redox compound, maintained lens GSH levels close to normal.

Effects of Molecular Oxygen on Diquat- and Paraquat-induced Damages to the Rabbit Lens in vitro

In the normal lens incubated in KR medium with HEPES buffer and glucose for 3 h in 100 % O_2 as the gas phase, MDA was 2.03 nmoles/g wet wt (Figure 3A) and GSH was 8.36 μmoles/g wet wt (Figure 3B). In the presence of 1 mM diquat or paraquat,

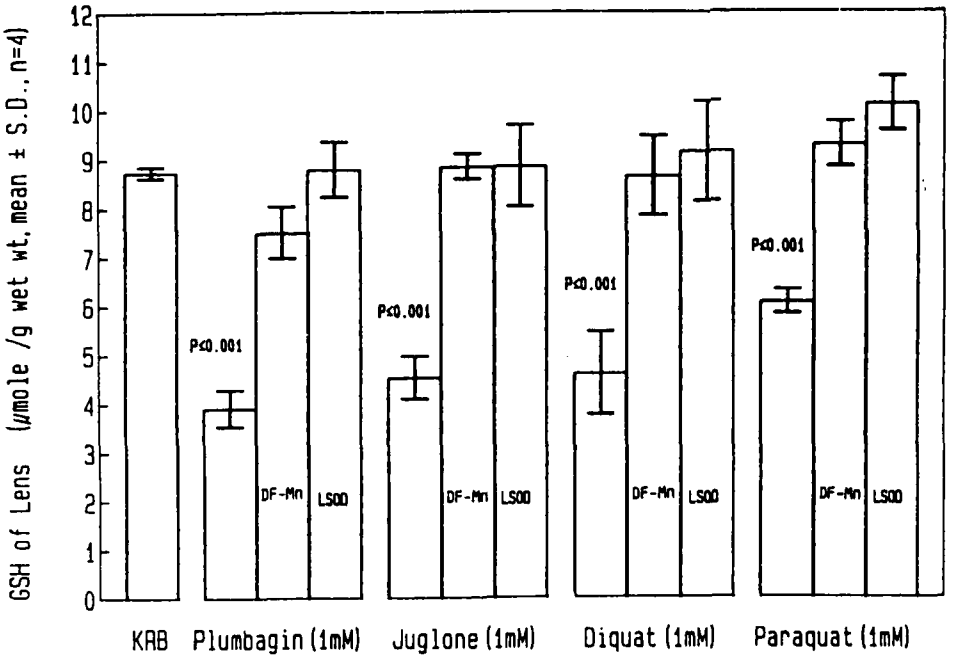

FIGURE 2B The effects of DF-Mn and LSOD on oxidation of GSH of the rabbit lens induced by redox compounds *in vitro*. The procedure of lens incubation was the same as described in the legend to Figure 2A. At the end of incubation, lenses were analyzed for GSH as described under "Materials and Methods" in the text.

lens MDA was higher ($P < 0.001$) 5 fold, and GSH was lower ($P < 0.001$) by 37–52% as compared to controls. The data given in Figure 3A and 3B also show that in normal lens incubated under similar experimental conditions but in the atmosphere of N_2, MDA was 0.83 nmole/g wet wt and GSH was 9.05 μmoles/g wet wt. In the presence of diquat or paraquat under anaerobic condition these lens constituents were not significantly altered.

Cataracts induced in Rabbits by Diquat in vivo

Diquat administered intravitreally as a single injection of 300 nmoles (30 μl) in the right eye of a rabbit, induced cataract (Figure 4A). The early signs of cataract, such as separation of anterior and posterior lens sutures with appearance of vacuoles, were visible by slit lamp 24–72 h after the injection. In 3–4 weeks the cataract advanced showing dense opacities (Figure 4A). The lens of the contralateral control eye was unchanged (Figure 4B).

The diquat and its free radical levels in the eye tissues, one week after the intravitreal injection of the compound, were determined spectrophotometrically, and calculated using $\varepsilon_{310\,nm}$ diquat $= 1.92 \times 10^4 \times M^{-1} \times cm^{-1}$ and $\varepsilon_{375\,nm}$ diquat free radical $= 2.8 \times 10^4 \times M^{-1} \times cm^{-1}$. The concentrations of diquat were 27.61 ± 3.72 nmoles/lens (Mean ± S.D., $n = 6$ eyes) and 32.85 ± 3.52 nmoles/vitreous humor of one eye ($n = 5$), and those of diquat free radical were

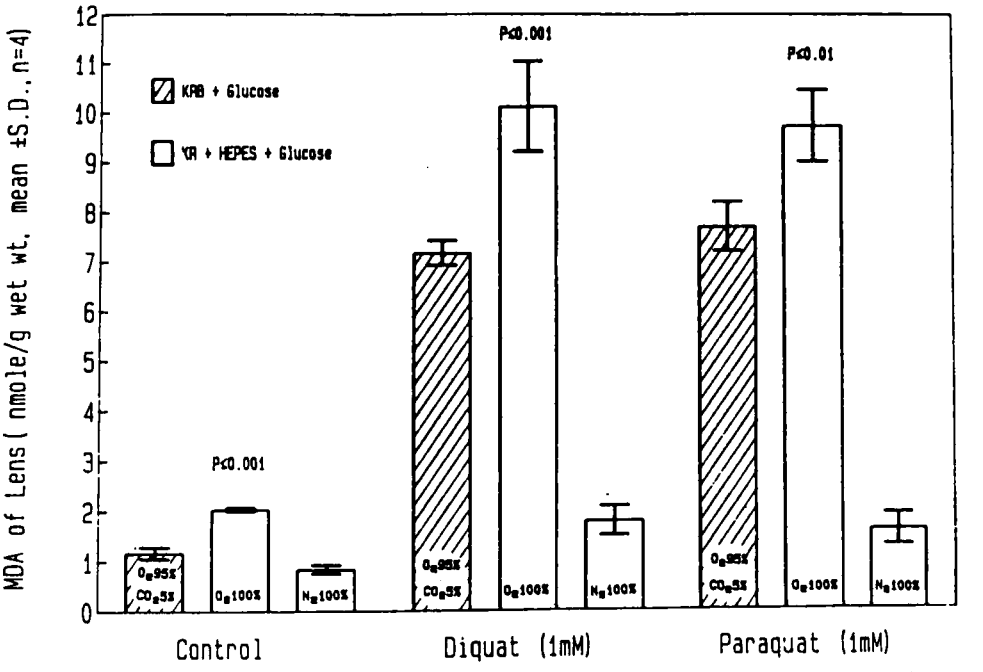

FIGURE 3A The effects of molecular oxygen on lipid peroxidation of the rabbit lens, induced by bipyridylium compounds *in vitro*. The lens incubation system and conditions were the same as described in the legend to Figure 2A, except Krebs-Ringer (KR) HEPES buffer, pH 7.4 was used when the gas phase was 100% O_2 or N_2.

6.99 ± 1.08 nmoles/lens ($n = 6$) and 2.59 ± 0.21 nmoles/vitreous humor of one eye ($n = 5$).

Reactive Species of O_2 in the Eye

Although it is not possible to measure the steady state levels of these extremely short-lived free-radical species of O_2, we were able to measure their rates of production over a period of 15 minutes for $O_2^{\cdot -}$ and 30 minutes for OH· under the specified assay conditions described in the "Materials and Methods" section.

In normal eyes of rabbits $O_2^{\cdot -}$ was 0.15 ± 0.03 μM (Mean ± S.D., $n = 6$ eyes) in the aqueous humor, 0.20 ± 0.05 μM in the vitreous humor, and was 4.86 ± 4.01 nmoles/g wet wt in the lens ($n = 3$) and 4.58 ± 4.57 nmoles/g wet wt in the retina ($n = 3$). In the diquat-injected right eye, after a week, $O_2 \cdot^-$ was higher 16 fold ($P < 0.001$) in the aqueous humor and vitreous humor, and 5 fold ($P < 0.01$) in the lens and retina as compared to the levels observed in the respective tissues of the contralateral control eye (Table I). In normal eyes ($n = 3$), OH· was not detectable in the aqueous humor, it was 45.71 ± 7.55 μM in the vitreous humor, 3.99 ± 2.67 nmoles/g in the lens and 273 ± 40 nmoles/g in the retina. In the early cataract OH· was higher 35 fold ($P < 0.001$) in the aqueous humor, 2 fold ($P < 0.05$) in the vitreous humor and 5 fold in the lens ($P < 0.05$) and retina ($P < 0.01$) as compared to normal eyes (Table I). In normal eyes ($n = 3$), H_2O_2 was

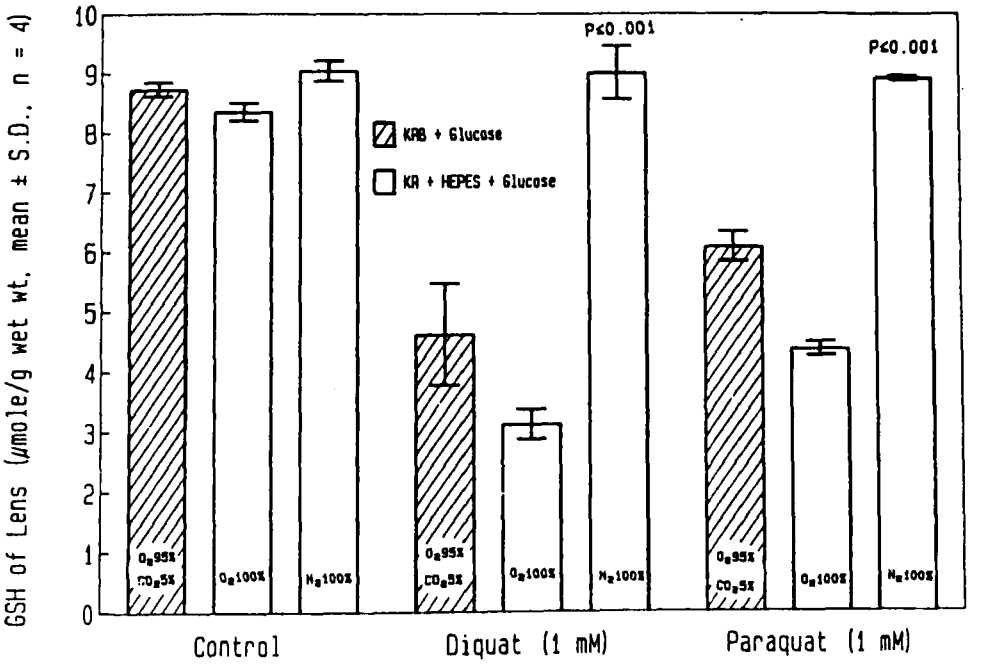

FIGURE 3B The effects of molecular oxygen on oxidation of GSH of the rabbit lens induced by bipyridylium compounds *in vitro*. After incubation of the lenses as described in the legend to Figure 3A., GSH was estimated according to the method described under "Materials and Methods" in the text.

TABLE I
Oxy Radicals and H_2O_2 in the Eye Tissues of Rabbit having Diquat-induced Early Cataract

Eye Tissues	Levels of the Reactive Species of O_2 (Mean ± S.D., n = number of eyes)	
	Control	Experimental
Aqueous humor		
$O_2^{\cdot-}$, μM	0.94 ± 0.08 (4)	15.09 ± 3.45 (4)[a]
$OH^{\cdot}$, μM	5.69 ± 4.09 (3)	199.15 ± 21.28 (3)[a]
H_2O_2, μM	33.00 ± 8.00 (8)	115.00 ± 11.0 (8)[a]
Vitreous humor		
$O_2^{\cdot-}$, μM	0.74 ± 0.08 (4)	13.73 ± 3.12 (4)[a]
$OH^{\cdot}$, μM	81.57 ± 8.94 (3)	143.29 ± 23.74 (3)[b]
H_2O_2, μM	15.00 ± 6.00 (8)	51.00 ± 8.00 (8)[a]
Lens		
$O_2^{\cdot-}$, nmole/g	7.74 ± 1.50 (4)	39.68 ± 7.62 (4)[c]
$OH^{\cdot}$, nmole/g	8.90 ± 2.96 (3)	61.53 ± 23.92 (3)[b]
Retina		
$O_2^{\cdot-}$, nmole/g	59.05 ± 20.16 (4)	386 ± 68 (4)[c]
$OH^{\cdot}$, nmole/g	342 ± 9 (3)	1538 ± 483 (3)[c]

[a] $P < 0.001$, [b] $P < 0.05$, [c] $P < 0.01$, Control eye VS. Experimental, "t" test, one-tailed.

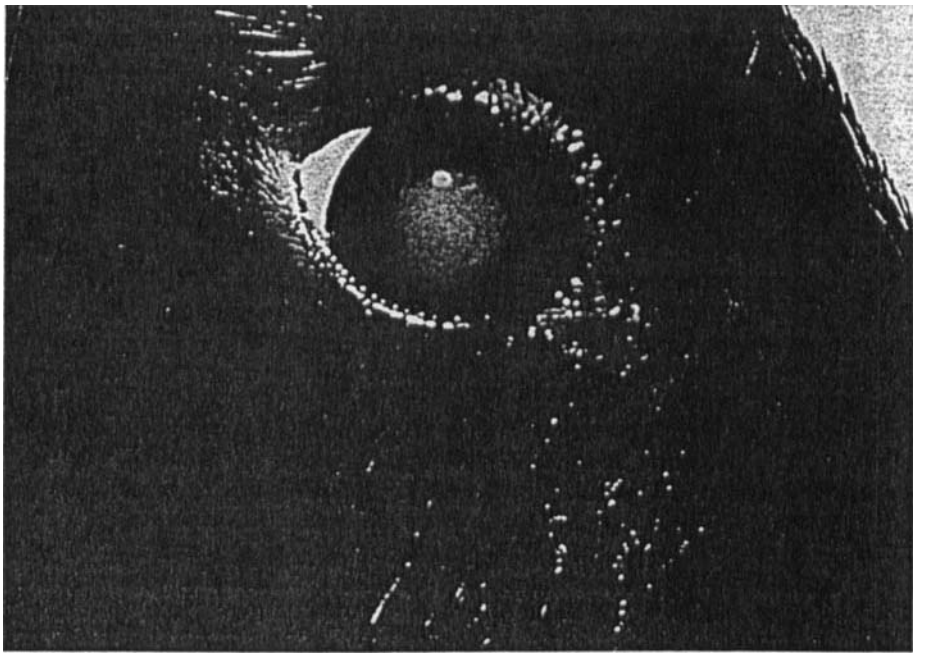

FIGURE 4A Diquat-induced cataract in a rabbit. An opaque lens showing a typical advanced cataract developed 4 wks after a single intravitreal injection of 300 nmoles (30 μl) diquat dibromide in the right eye.

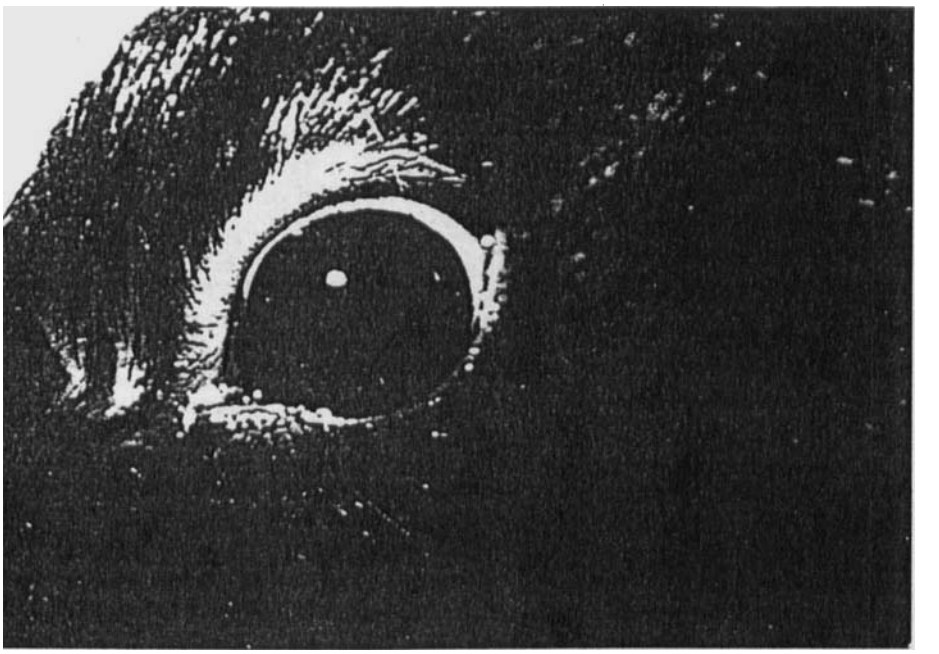

FIGURE 4B Contralateral control eye of the rabbit referred in Figure 4A. No opacity was observed in the lens of the left eye, 4 wks after a single intravitreal injection of 30 μl of physiological saline (145 mM NaCl).

$35 \pm 7\,\mu M$ in the aqueous humor and $20 \pm 4\,\mu M$ in the vitreous humor. These values are similar to those previously reported.[11,14,19] In the early cataract H_2O_2 was higher ($P < 0.001$, $n = 8$) 4 fold in the aqueous humor, and 3 fold in the vitreous humor as compared to the controls (Table I).

DISCUSSION

Experimental evidence from these studies demonstrates for the first time the direct participation of O_2 free radicals in cataractogenesis. A variety of foreign compounds such as plumbagin and juglone which readily accept one electron, forming their respective semiquinone free-radical intermediates, and diquat and paraquat, producing monocationic free radicals, cause oxidation of GSH and peroxidation of cellular membrane lipids of lens *in vitro*. Their deleterious effects on the lens, are promoted by O_2, arrested by N_2, and inhibited by LSOD or the SOD-mimic, DF-Mn. Thus, it is evident that $O_2^{\cdot-}$ is the inducer of toxic effects of these compounds on the lens *in vitro*. Such an effect of O_2 in potentiating the paraquat toxicity to *Escherichia coli* in culture, is reported.[3] There are several reports showing that the free radical species produced by the univalent reduction of xenobiotics readily react with dioxygen generating superoxide and the original compounds.[1-4] Therefore, in cells with the availability of an ample endogenous source of reducing equivalents, minute quantity of such a redox compound can generate $O_2^{\cdot-}$ in excessive amounts by cyclic oxidation reduction reactions.

Diquat administered as a single intravitreal dose of 300 nmoles in one eye of a rabbit induces early cataract after 24–72 h, and the lens of the contralateral physiological saline-injected eye is unchanged. The cataract progresses to maturity after 3–4 wk, is always associated with retinal degeneration, and in the majority of rabbits a band-shaped keratopathy is also observed in corneas of the diquat-injected eyes. Light microscopic examination of the experimental eyes reveals no signs of inflammation. Our findings confirm the previous reports[6,7] on the cataractogenic effect of diquat in animals in which bilateral cataracts were induced in rats fed on a diet containing diquat (0.035–0.075 %) for 4–6 months, and in dogs fed on diquat (15 mg/kg body wt/day) for a year.[6] It is suggested that endogenous redox system consisting of NADPH and glutathione reductase reacting with diquat could give rise to diquat free radical and H_2O_2 which might involve in the mechanism of diquat-induced cataract in rats.[9]

The most important observation in our study is that in the diquat-injected eye of the rabbit having early cataract, concentrations of the reactive species of O_2 in eye tissues, were significantly higher than in the controls. The $O_2^{\cdot-}$ was about 16 fold higher in the aqueous humor and vitreous humor and about 5 fold higher in the lens and retina, $OH\cdot$ was higher 35 fold in the aqueous humor, 2 fold in the vitreous humor and about 5 fold in lens and retina as compared to the control eyes. H_2O_2 was 3–4 fold higher in the aqueous humor and vitreous humor than in the controls. The levels of $O_2^{\cdot-}$, $OH\cdot$, and H_2O_2 in the tissues of the control eye, were close to normal.

Under similar experimental conditions investigation of the status of endogenous redox systems in the eye with diquat-induced cataract shows marked decreases in the ascorbic acid of the aqueous humor, vitreous humor and lens, impairment of enzymatic defences in the lens against reactive species of O_2, and a small but significant fall in the lens GSH with no change in soluble protein-SH. Enhanced peroxidation of

cellular membrane lipids and formation of aminophospholipid·MDA adduct also occur in diquat-induced cataract (Data not included). Similar peroxidative damages are reported in the human senile cataract.[20-23]

We propose that redox active compounds such as diquat, by cyclic reduction and oxidation reactions coupled to the endogenous redox systems of the eye, may divert the flow of electrons to the molecular oxygen generating excessive amount of O_2^- which forms H_2O_2 and OH· triggering cataractogenesis.

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