Fluorophotometric Detection of Intravitreal Peroxides after Panretinal Laser Photocoagulation

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anretinal laser photocoagulation (PRP) has been a mainstay in the treatment of proliferative diabetic retinopathy and other retinal ischemic diseases, such as central retinal vein occlusion. The beneficial effect of PRP has not been fully elucidated, but it has been thought to result from the following: a decrease in oxygen consumption by destroying outer retina, especially photoreceptors that have high oxygen consumption; the elimination of angiogenic factors by ablating ischemic retina; the induction of retinal pigment epithelial cells to secrete inhibitor of vascular endothelial cell proliferation; and the increase in the level of retinal oxygenation by diffusing oxygen from the choroid. In addition, previous experimental and clinical studies have reported that PRP can cause posterior vitreous detachment by inducing vitreous liquefaction. The development of complete posterior vitreous detachment reportedly prevented the progression of neovascularization in proliferative diabetic retinopathy; although vitreous liquefaction alone is not enough to cause complete posterior vitreous detachment, PRP may be associated with the change in vitreoretinal interface, which can influence the prognosis of diabetic retinopathy.

METHODS. In the presence of \( \text{H}_2\text{O}_2 \) and lipid hydroperoxides, nonfluorescent DCFH was oxidized to highly fluorescent \( 2',7' \)-dichlorofluorescein (DCF; excitation, 495 nm; emission, 520 nm), which is detectable by vitreous fluorophotometry. Reactions of DCFH, including hematin with various concentrations of \( \text{H}_2\text{O}_2 \), were investigated in vivo. Fluorophotometry with DCFH was performed 1, 7, 14, and 28 days and 2 months after argon laser PRP. Untreated eyes served as the controls.

RESULTS. Exogenously applied \( \text{H}_2\text{O}_2 \) oxidized DCFH to DCF in a dose-dependent manner, ranging from \( 6 \times 10^{-6} \text{ mol/l} \) to \( 6 \times 10^{-7} \text{ mol/l} \) in concentration in vivo. Intravitreal DCF concentration was 83.7 ± 6.8 nmol/l in control eyes. A significant increase of DCF was detected 1 day after PRP (330.7 ± 123.8 nmol/l, \( P < 0.002 \)). The increase peaked on day 7 (352.4 ± 239.5 nmol/l, \( P < 0.002 \)) and remained elevated at 2 months after PRP (161.8 ± 51.4 nmol/l, \( P < 0.01 \)).

CONCLUSIONS. This method allowed a highly sensitive quantitation of intravitreal peroxides in vivo. The authors’ findings indicated that PRP induces increased production of peroxides in rabbit vitreous for 2 months. The data suggested that persistently high levels of peroxides in the vitreous humor affect the development of vitreous liquefaction after PRP. (Invest Ophthalmol Vis Sci. 1998; 39:358-363)
Materials and Methods

The following materials were used in the study: DCFH diacetate (Molecular Probes, Eugene, OR); DCF (Lambda, Grottendhofs, Austria); hematin (Sigma Chemical, St. Louis, MO); and 30% H$_2$O$_2$ (Wako Chemical, Tokyo, Japan).

The hematin stock solution was prepared by dissolving 0.05 g hematin in 25 ml 0.2 N sodium hydroxide. This solution remains stable for 3 months when refrigerated. The 0.5-ml hematin stock solution was diluted to 50 ml of 25 mM sodium phosphate buffer (pH 7.4) and then purged with argon gas on ice. DCFH was prepared from DCFH diacetate after the method described previously, with some modifications. In brief, immediately before the fluorophotometric measurements, the hydrolysate of DCFH diacetate, 0.5 ml, was neutralized with 12 ml of 25 mM sodium phosphate buffer containing 20 µg/ml hematin. The final concentrations of DCFH and hematin were 40 µM and 19.2 µg/ml, respectively.

The measurements of each fluorescent intensity were performed on a scanning fluorophotometer (Fluorotron Master; OcuMetrics, Mountain View, CA). Fluorescent intensity was linearly related to a DCF concentration between 1 nmol/l and 10,000 nmol/l with the fluorophotometer. DCF concentration was calculated from the DCF fluorescence with a standard calibration curve.

Animals and Anesthesia

Thirty-nine Dutch rabbits, weighing between 2.5 and 2.8 kg each, were used for the in vivo study. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were divided into four groups: group A (four eyes), to examine the intravitreal kinetics of the dye with hematin; group B (16 eyes), to determine the sensitivity of which H$_2$O$_2$ in the presence of hematin can oxidize DCFH to DCF in the vitreous cavity; group C (38 eyes), to measure intravitreal peroxides after PRP and to compare them with those of untreated control eyes; and group D (20 eyes), to estimate the influence of PRP on intravitreal kinetics of the dye. Before the preinjection scan, the rabbits were anesthetized with an intramuscular injection of ketamine (5 mg/kg) and xylazine (2 mg/kg). Additional anesthesia was administered every 30 minutes if required. The pupils were maximally dilated with 10% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride. To ensure proper axial alignment of the eye, each rabbit was kept in a semicylinder-shaped holder with its head fixed only during the fluorophotometric measurement, which was done within a few minutes.

Intravitreal Kinetics of 2',7'-Dichlorofluorescein with Hematin (Group A)

To investigate whether the addition of hematin into DCFH solution would influence the intravitreal kinetics of DCFH, DCF solution, including hematin, was substituted and injected into the vitreous, and the fluorescence of DCF was monitored for 2 hours. After a preinjection scan, paracentesis was made with a 1-mm slit knife, and 0.1 ml of 40 µM DCF solution, including 19.2 µg/ml hematin, was injected into the vitreous cavity with a 27-gauge needle inserted at the pars plana. Fluorophotometry was performed immediately and at 5, 10, 20, 30, 45, 60, 90, and 120 minutes after injection. DCF fluorescence and DCF concentration in the vitreous were defined as described previously. The elimination constant rate after 2 hours was calculated by dividing the DCF concentration at 60 minutes by that at 120 minutes.

H$_2$O$_2$-Dependent Oxidation of 2',7'-Dichlorofluorescein in the Presence of Hematin in Vivo (Group B)

After a preinjection scan and paracentesis were performed, 0.1 ml DCFH–hematin solution was injected into the rabbit vitreous. Intravitreal administration of 0.1 ml H$_2$O$_2$, in various concentrations (10$^{-6}$ to 10$^{-3}$ mol/l), and the fluorophotometric measurements were performed as described previously. For each concentration of H$_2$O$_2$, four eyes were used. The H$_2$O$_2$-dependent DCF production was determined by subtracting the DCF concentration at 60 minutes after the DCFH injection (without H$_2$O$_2$) from that at 60 minutes after the H$_2$O$_2$ injection.

Intravitreal Peroxides after Panretinal Laser Photocoagulation to the Retina (Group C)

Under anesthesia, argon laser PRP was performed on 28 eyes of 14 rabbits with the use of a slit lamp and a panfunduscope. A total of 350 moderate burns was applied to each eye under the following conditions: spot size, 500 µm; exposure time, 0.2 second; and laser power, 200 mW to 300 mW. The power settings were adjusted to obtain a grade 2 to grade 3 lesion (whitish center surrounded by one or two grayish rings). The posterior half of the entire retina was coagulated. Ophthalmoscopic examinations were made just after the treatment, and eyes with either vitreous or retinal hemorrhage were excluded from the experiments. Vitreous fluorophotometry was performed at 1 (six eyes), 7 (six eyes), 14 (six eyes), and 28 days (six eyes) and 2 months (four eyes) after PRP. For fluorophotometry, 0.1 ml DCFH–hematin solution was injected into the vitreous cavity after preinjection imaging, and paracentesis was conducted. A measurement was recorded 60 minutes after DCFH injection. Ten eyes of five other rabbits served as the untreated normal controls. The DCF production in PRP-treated eyes was compared with that of untreated control eyes using the Mann–Whitney test.

Intravitreal Kinetics of 2',7'-Dichlorofluorescein after Panretinal Laser Photocoagulation (Group D)

To estimate the influence of PRP on intravitreal kinetics of the dye, PRP was performed on 20 eyes and vitreous fluorophotometry with DCF was performed, as described in group A, at 1, 7, 14, and 28 days and 2 months after PRP. Four eyes were used for each time point. The elimination constant rates after 2 hours for each time point were statistically compared with those of untreated control eyes (obtained in group A) using the Mann–Whitney test.

Results

Intravitreal Kinetics of 2',7'-Dichlorofluorescein with Hematin (Group A)

DCF uniformly diffused into the vitreous cavity within 60 minutes after injection, and its concentration was maintained...
for at least 2 hours (Fig. 1). The elimination constant rate after 2 hours represented 18.56 ± 16.71%.

H$_2$O$_2$-Dependent Oxidation of 2',7'-Dichlorofluorescin in the Presence of Hematin in Vivo (Group B)

An initial scan, which was measured 60 minutes after administration of DCFH alone (without H$_2$O$_2$), revealed a mild increase in vitreal fluorescence. H$_2$O$_2$ oxidized DCFH to fluorescent DCF dose dependently, in the range of 6 × 10$^{-8}$ mol/l to 6 × 10$^{-5}$ mol/l, assuming that the volume of rabbit vitreous was 1.47 ml$^3$ (Fig. 2). The range was similar to that found in the in vitro experiment.$^{39}$

Intravitreal Peroxides after Panretinal Laser Photocoagulation to the Retina (Group C)

In untreated control eyes, preinjection imaging revealed normal autofluorescence of cornea, lens, and choroid-retina (Fig. 3A, dotted lines). The scan, performed 60 minutes after DCFH injection, detected a mild increase in DCF concentration in the posterior vitreous (Fig. 3A, solid lines). Figure 3B shows typical fluorophotometric scans registered 1 day after PRP. The preinjection scan (before DCFH injection) showed a minimal increase in vitreal fluorescence (dotted lines). The scan performed 60 minutes after DCFH injection demonstrated increased DCF production in the posterior vitreous (solid lines). In untreated control eyes, intravitreal DCF concentration was 83.7 ± 6.8 nmol/l. A significant increase of DCF was detected 1 day after PRP (330.7 ± 123.8 nmol/l, $P < 0.002$). The increase peaked on day 7 (352.4 ± 239.5 nmol/l, $P < 0.002$) and remained elevated at day 14 (260 ± 39.1 nmol/l, $P < 0.002$), day 28 (163 ± 46.5 nmol/l, $P < 0.005$), and even at 2 months after PRP (161.8 ± 51.4 nmol/l, $P < 0.01$) (Fig. 4).

DISCUSSION

We have developed a highly sensitive method for in vivo quantitation of intravitreal peroxides, at the nanomole level, by vitreous fluorophotometry with DCFH and hematin. The addition of hematin provided a 1000-fold more sensitive evaluation for intravitreal peroxides in the living eye than previously reported.$^{36}$ Hematin, possibly by accelerating the reaction between hydroperoxides and DCFH, could heighten the sensitivity. It has been reported that hematin has peroxidase activity.$^{40}$ The most likely mechanism is one in which hematin forms a hematin-peroxide complex, which degrades to a ferryl-oxo compound and an hydroxyl radical, both of which are capable of oxidizing DCFH more rapidly.$^{41}$ Thus, although hematin increased the sensitivity of the assay of peroxides, overestimation may be a possibility and the obtained level may not be the real value. However, this modified method with hematin has the advantage of detecting small changes in the level of peroxides in the living vitreous.

Although adding hematin to the DCFH solution and injecting the mixture into vitreous may influence the kinetics of the...
of hematin alone did not affect the baseline fluorescence of retina and vitreous (data not shown). Hence, we suggest that the use of hematin has little influence on oxidative status in the vitreous. Because any toxicity data for DCFH have not been thoroughly investigated, it will be difficult to use this technique for clinical diagnostic purposes in humans. However, it will be important as an experimental technique to study the oxidative status in the eye in some pathologic conditions.

Vitreous fluorophotometry disclosed a significant increase in DCF production 1 day after PRP. The increase peaked on day 7 and remained elevated even at 2 months. Because the findings in group D revealed that there was no statistically significant difference in outward movement of DCF after PRP, the influence of PRP regarding outward movement of the dye is considered negligible, so long as the scan is performed 1 hour after injection. Few studies have been concerned with the increased production of reactive oxygen species in the vitreous humor after PRP. It is plausible, in the acute phase, that both thermal and photochemical effects of laser energy directly cause lipid peroxidation of rod outer segment polyunsaturated fatty acids, which are susceptible to peroxidation. Moreover, photoradiation of melanin pigments, which are rich in retinal pigment epithelial and choroid, under aerobic conditions can generate reactive oxygen species. Furthermore, inflammatory cell infiltration may contribute to the additional retinal peroxidation. This retinal peroxidation could bring about subsequent cell membrane damage, which may result in an efflux of lipid hydroperoxides to the vitreous cavity. Delayed infiltration of inflammatory cells into the vitreous cavity also might have induced peroxidation of the vitreous, which includes moderate amounts of docosahexaenoate. However, all these reactions are inadequate for accounting for the long-standing high level of intravitreal peroxides, because direct thermal-photochemical and subsequent inflammatory damages are temporary and subside within 2 to 4 weeks at the most. It is, therefore, speculated that PRP might have produced permanent changes in the

dye, the results in group A indicated that DCF uniformly diffused into the vitreous within 60 minutes and was maintained for at least 2 hours after injection (Fig. 1). Therefore, we suggest that the addition of hematin has little influence on vitreal concentration and on the outward movement of DCF, insofar as the measurement is performed within 60 minutes after injection. In turn, the data from untreated normal control eyes in group C demonstrated a mild increase in DCF production in the vitreous (Fig. 3A). The increase may have resulted from the oxidation of DCFH by background peroxides, which are normally present in the vitreous humor. A minor inflammation caused by injection of the dye may generate additional peroxides, which can also oxidize DCFH. The obtained DCF concentration was $83.7 \pm 6.8 \text{ nmol/l}$, approximately equivalent to $10^{-3} \text{ mol/l H}_2\text{O}_2$, according to the data represented in Figure 2. The data are consistent with those reported by Bhuyan and Bhuyan. Moreover, intravitreal administration
oxidative state of vitreous. An increased oxygen supply from the choroid through the laser scar into vitreous may be one of the changes.²⁻⁸⁻¹¹

Previous experimental studies have shown that vitreous liquefaction could not be observed immediately but occurred at least 1 to 2 months after laser treatment.¹²⁻¹³ The acute effects of photocoagulation, such as thermal and photochemical damage, inflammation, and influx of low-molecular proteins into the vitreous subsequent to disruption of the blood–ocular barrier, are insufficient for explaining the delay in the development of vitreous liquefaction. However, group C data indicated that increased production of intravitreal peroxides, which were capable of degrading hyaluronate, were maintained for at least 2 months. Although changes of vitreous structure were not investigated in this study because of in vivo fluorophotometric measurement, experimental vitreous liquefaction reportedly occurred after photocoagulation under milder conditions.¹²⁻¹³ The persistently high levels of intravitreal peroxides demonstrated herein may relate to the delayed development of vitreous liquefaction after PRP.

In conclusion, we have developed a highly sensitive method for the in vivo quantitation of intravitreal peroxides by vitreous fluorophotometry. With the use of this method, we showed that PRP induced the increased production of peroxides in rabbit vitreous for 2 months. The data suggested that persistently high levels of peroxides in the vitreous humor affect the development of vitreous liquefaction after PRP.

References


