Rapid Diffusion of Hydrogen Protects the Retina: Administration to the Eye of Hydrogen-Containing Saline in Retinal Ischemia-Reperfusion Injury

Running title: **Protective effect of H_2 against retinal I/R injury**

Hideaki Oharazawa,[1] Tsutomu Igarashi,[2] Takashi Yokota,[3] Hiroaki Fujii,[1] Hisaharu Suzuki,[2] Mitsuru Machide,[4] Hiroshi Takahashi,[2] Shigeo Ohta,[5] and Ikuroh Ohsawa[4]

From [1]Department of Ophthalmology, Musashikosugi Hospital, Nippon Medical School, Kawasaki City, Kanagawa 211-8533, Japan; [2]Department of Ophthalmology, Nippon Medical School, Bunkyo-ku, Tokyo 113-8603, Japan; and [3]Department of Molecular Biology, [4]The Center of Molecular Hydrogen Medicine and [5] Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Nippon Medical School, Kawasaki City, Kanagawa 211-8533, Japan.

Supported in part by Ministry of Education, Culture, Sports, Science, and Technology of Japan Grants-in-Aid (19659331 to S. O. and 20500345 to I. O.).

Corresponding author: Ikuroh Ohsawa, The Center of Molecular Hydrogen Medicine, Institute of Development and Aging Sciences, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki, 211-8533 Japan. iohsawa@nms.ac.jp.

Abstract

PURPOSE. Retinal ischemia-reperfusion (I/R) injury by transient elevation of intraocular pressure (IOP) is known to induce neuronal damage through the generation of reactive oxygen species. Previous studies indicate that molecular hydrogen (H_2) is an efficient antioxidant gas that selectively reduces the hydroxyl radical (•OH) and suppresses oxidative stress-induced injury in several organs. This study was conducted to explore the neuroprotective effect of H_2-loaded eye drops on retinal I/R injury.

METHODS. Retinal ischemia was induced in rats by raising IOP for 60 minutes. H_2-loaded eye drops were prepared by dissolving H_2 gas into a saline to saturated level and administered to the ocular surface continuously during the ischemia and/or reperfusion periods. One day after I/R injury, apoptotic cells in the retina were quantified and oxidative stress was evaluated by markers such as 4-hydroxynonenal and 8-hydroxy-2-deoxyguanosine. Seven days after I/R injury, retinal damage was quantified by measuring the thickness of the retina.

RESULTS. When H_2-loaded eye drops were continuously administered, H_2 concentration in the vitreous body immediately increased and I/R-induced •OH level decreased. The drops reduced the number of retinal apoptotic and oxidative stress marker-positive cells, and prevented retinal thinning with an accompanying activation of Müller glia, astrocytes, and microglia. The drops improved the recovery of retinal thickness by >70%.

CONCLUSIONS. H_2 has no known toxic effects on the human body. Thus, our study suggests that H_2-loaded eye drops will be a highly useful neuroprotective and anti-oxidative therapeutic treatment for acute retinal I/R injury.

INTRODUCTION

Retinal ischemia-reperfusion (I/R) injury by transient elevation of intraocular pressure (IOP) in animal models is known to induce necrosis and apoptosis of cells and significant reductions in thickness in multiple layers of the retina.[1, 2] Clinically, these features closely resemble a number of diseases such as acute angle-closure glaucoma, retinal artery occlusion and amaurosis fugax.[3] It can irreversibly damage the retina, causing visual impairment and blindness. Immediate mechanisms of I/R injury involve the formation of reactive oxygen species (ROS),[4] which has been considered to contribute to the pathogenesis of many neurodegenerative diseases including glaucomatous neurodegeneration.[5] Endogenous antioxidant enzymes and organic free radical scavengers can retard or prevent neuronal damages of retinal I/R injury in many animal models.[6-13] One highly reactive ROS, hydroxyl radical (•OH), is generated during the early phase of reperfusion after ischemia and a major cause of retinal injury.[14-16] •OH attacks lipids, proteins and nucleic acids causing irreversible cellular damage.

In the past two decades, much attention has been focused on the use of several pharmaceutical gaseous molecules to attenuate oxidative stress.[17] A variety of gas delivery systems are used and under development for safe and effective administration of medical gases. We have previously reported that H_2 selectively reduces •OH and peroxynitrite without affecting other oxygen-derived free radicals.[18] Inhalation of H_2 gas has been demonstrated to limit the infarct volume of the brain, heart and liver by reducing I/R injury[18-21] and can ameliorate intestinal transplant injury.[22] Moreover, the consumption of water with dissolved H_2 to a saturated level prevents stress-induced cognitive decline and 6-hydroxydopamine-induced nigrostriatal degeneration.[23,24] One clinical trial demonstrated a decrease in low-density lipoprotein after drinking H_2-loaded water.[25] H_2 has the potential to easily diffuse into organs and no known toxic effects on the human body.[18]

We have therefore developed a simple and effective method to deliver H_2 into lesions. The method is H_2-loaded eye drops, which are convenient, compared with the inhalation of H_2 gas, for the treatment of eye diseases. In this paper, we demonstrate that the continuous administration of H_2-loaded eye drops immediately increases H_2 concentration in the vitreous body and prevents I/R-induced oxidative stress, leading to a decrease in apoptotic cell death in the retina and a decrease in retinal thinning with glial responses.

METHODS

Administration of H_2 and Measurement of its Concentration

H_2-loaded eye drops were prepared by bubbling H_2 gas (flow rate: 1 l/min) through 400 ml of normal saline solution with stirring for 10 min to a saturated level (Fig. 1A), and then stored in an aluminum foil bag (see Fig. 1B, Hosokawa Yoko, Tokyo, Japan) with no dead volume. The concentration of H_2 in the bag slowly decreased with a half-life of approximately 3 months. Freshly prepared H_2-loaded eye drops were administered to the ocular surface continuously (4 ml/min) with a dropper connected to aluminum foil bag during the ischemia and/or reperfusion periods. The H_2 dissolved in saline solution was measured using a needle-type H_2 sensor (Unisense, Aarhus N, Denmark). To measure the H_2 concentration on the ocular surface, the sensor was touched to the surface. To measure the H_2 concentration in the vitreous body, the sensor was inserted into the vitreous body through the sclera.

To investigate the effect of H_2-loaded eye drops on retinal I/R injury, we applied them using four different time courses (see Fig. 4A). Duration F: Eye drops with and without H_2 were applied during an entire 90 min process (60 min of ischemia followed by 30 min of reperfusion). Duration I: Eye drops with H_2 were applied only during ischemia. Duration R: Eye drops with H_2 were applied only after reperfusion. Duration I/R: Eye drops with H_2 were applied for 10 min before and 30 min after reperfusion.

Induction of I/R Injury

Retinal I/R injury was induced essentially as described previously.[2,26] Seven-week-old male Sprague-Dawley rats weighing 200 to 250 g were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg), and the pupils were dilated with topical phenylephrine hydrochloride and tropicamide. After topical application of 0.4% oxybuprocaine hydrochloride,

the anterior chamber was cannulated with a 30-gauge infusion needle connected to a normal saline reservoir. The IOP was raised to 110 mmHg for 60 min by elevating the saline reservoir. Body temperature was maintained at 37.0 ± 0.5 ºC with a rectal thermometer probe and a heating pad during the experimental period. Retinal ischemia was confirmed by whitening of the iris and fundus. After 60 min of ischemia, the needle was withdrawn from the anterior chamber and the intraocular pressure was normalized. The animals were euthanized with an overdose of anesthesia after reperfusion, and the eyes were immediately enucleated. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The studies were approved by the Animal Care and Use Committee of Nippon Medical School. All experiments were performed by examiners blinded to the genotypes or treatments of the rat.

Detection of •OH

The procedure for the measurement of accumulated •OH in the eye is similar to that previously described with modifications.[27] We used 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl] benzoate (HPF; DAIICHI PURE CHEMICALS, Tokyo, Japan), which detects highly reactive ROS including •OH, as a fluorescence probe.[28] HPF (4 μl, 1 mM) was given intravitreally just before the induction of ischemia. Rats were killed after 60 min of ischemia followed by 15 min of reperfusion. Retinas were quickly removed and flat mounted without fixation. The fluorescence images were acquired using a laser-scanning confocal microscope. The acquired images were analyzed by quantitative comparisons of the relative fluorescence intensity of retinas between groups using NIH Image software.

Histopathologic and Morphometric Study

Eyes were enucleated 7 days after reperfusion and fixed in 1% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) for 30 min, and the anterior segments were removed. Corneas and lenses were discarded. The entire eye cups were further fixed in the same solution overnight and then transferred to 30% sucrose for cryo-protection. Cryosections (10 μm thick) were cut along the vertical meridian of the eye, passing through the optic nerve head, and were stained with hematoxylin and eosin (H&E). Retinal damage was assessed by measuring the thickness of the retina.[1] The thickness is defined as the total width between the inner limiting membrane to the interface of the outer plexiform layer and the outer nuclear layer. These measurements were made at a distance within 1-2 mm from the optic disc using a light microscope. The value was averaged from four measurements in the temporal and nasal hemispheres of three different sections.

TUNEL Assay and Immunohistochemical Staining

One or seven days after reperfusion, the eyes were immediately enucleated. For TUNEL assay and the staining of reactive gliosis markers they were fixed in 4% PFA, and for staining of oxidative stress markers they were fixed in Bouin's fluid for 30 min. Next, the anterior segments were removed and the corneas and lenses were discarded. For the TUNEL assay and for the staining of reactive gliosis markers, the obtained entire eye cups were further fixed in the same solution overnight; for the staining of oxidative stress markers, they were further fixed in the same solution for 2 hr. After cryo-protection with 30% sucrose, cryosections (10 μm thick) were cut along the vertical meridian of the eye, passing through the optic nerve head. TUNEL staining was performed using an apoptosis detection kit according to the supplier's instructions (CHEMICON, Norcross, GA).[29] The numbers of TUNEL-positive cells in the retina were counted at a final magnification of x200 for each section using a light microscope.

For the immunostaining of oxidative stress markers, 4-hydroxynonenal (4-HNE) and 8-hydroxy-2-deoxyguanosine (8-OHdG),[30,31] cryosections were postfixed in acetone and stained using the ABC kit according to the supplier's instructions (Vector Laboratories, Burlingame, CA).[18] Sections were incubated with the following primary antibodies: Mouse monoclonal anti-4-HNE (1:400; JaICA, Shizuoka, Japan) and mouse monoclonal anti-8-OHdG (1:20; JaICA), in a blocking buffer for 1 hr at 4 ºC. The stained sections were further counterstained for nuclei with methyl green (0.5%). The numbers of 4-HNE- and 8-OHdG-positive cells in the retina were counted at a final magnification of x200 of each section using a light microscope.

For immunofluorescent staining of microglia and macroglia (astrocytes and Müller cells), cryosections were incubated with the following primary antibodies: Rabbit polyclonal anti-Iba1[32] (1:100; Wako, Osaka, Japan) or rabbit polyclonal anti-glial fibrillary acidic proteins (GFAP) (1:500; DAKO, Glostrup, Denmark) in blocking buffer for 1 hr at room temperature. After being washed twice with PBS, sections were

incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100; Invitrogen, Carlsbad, CA) for 30 min and further counterstained for nuclei with propidium iodide for 10 min. The numbers of Iba1-positive cells in the retina were counted at a final magnification of x200 of each section using a scanning laser confocal microscope (FV300, Olympus, Tokyo, Japan).

Statistical Analysis

We used StatView software (SAS Institute, Cary, NC) for statistical analysis. All statistical values are presented as the mean ± SD. For single comparisons, we performed an unpaired two-tailed Student's *t*-test. For multiple comparisons, we used an analysis of variance (ANOVA) followed by Fisher LSD test. A $P < 0.05$ was considered statistically significant.

RESULTS

H_2-Loaded Eye Drops Increase H_2 Concentration in the Vitreous Body and Reduce the Accumulation of •OH during Retinal I/R

We prepared a H_2-saturated normal saline solution (0.8 mM, pH 7.2; H_2-loaded eye drops) and packed it into an aluminum foil bag to prevent a decrease in H_2 concentration. A dropper connected to the bag was held close to the rat eye and drops were applied onto the ocular surface. The time-course of changes in H_2 levels was monitored using a needle-shape hydrogen sensor electrode inserted through the sclera to the vitreous body. When H_2-loaded eye drops were administered continuously, approximately 0.5 mM H_2 was detected on the ocular surface (Fig. 1C). Two minutes after the start of administration, H_2 concentration in the vitreous body started to increase and reached a maximum level after 15 min (Fig. 1D). At that time, the H_2 concentration accounted for approximately 20% (0.16 mM) of the H_2-loaded eye drops. Immediately after administration of the H_2-loaded eye drops ceased, the H_2 concentration in the vitreous body was observed to gradually decrease and then completely disappear after 15 min (Fig. 1D). The maximum observed concentration of H_2 in the vitreous body was approximately one third of the value observed on the ocular surface (Fig. 1E).

To verify that the diffused H_2 protects against •OH during retinal I/R, we assessed the accumulation of •OH by the fluorescence signal emitted by the oxidized form of HPF.[28] We produced retinal ischemia in rats by increasing IOP with an infusion needle connected to a saline bag. Just before the induction of ischemia, 4 μl of 1 mM HPF was given intravitreally, followed by 60 min of ischemia. Fifteen minutes after reperfusion, retinas were flat mounted and imaged in their entirety using a laser confocal-scanning microscope (Fig. 2A). The retinal HPF-fluorescence in the H_2-loaded eye drop-treated group was significantly less than that in the vehicle-treated group (Fig. 2B).

H_2-Loaded Eye Drops Decrease the Number of Apoptotic and Oxidative Stress Marker-Positive Cells

To determine whether the administration of H_2-loaded eye drops protects against retinal I/R injury, eye drops with and without H_2 were applied during the entire 90 min process (60 min of ischemia followed by 30 min of reperfusion). One day after I/R injury, a remarkable increase in the number of apoptotic cells (TUNEL-positive cells) was observed in both the inner and the outer nuclear layers of vehicle-treated retinas (Fig. 3A); however, the administration of H_2-loaded eye drops resulted in a significant decrease (about 77%, $P < 0.0001$) of TUNEL-positive cells (Figs. 3A and 3B), indicating that H_2-loaded eye drops had potent anti-apoptotic activity. We speculate that the decreased apoptotic cell death reflects the H_2-dependent reduction of oxidative stress, which was mainly promoted by •OH.

We then examined the levels of two oxidative stress markers, 4-HNE and 8-OHdG, in the vehicle-treated and the H_2-loaded eye drop-treated eyes by immunohistochemical staining with each specific antibody.[30,31] As expected,[9] one day after I/R injury, the number of 4-HNE and 8-OHdG-positive cells increased dramatically in the retina, respectively (Figs. 3C and 3E). However, eyes that had been treated with H_2-loaded eye drops exhibited significantly fewer 4-HNE and 8-OHdG-positive cells compared with the vehicle-treated retinas (Figs. 3C-3F), supporting the hypothesis formulated above.

H_2-Loaded Eye Drops Reduced Histopathological and Morphometrical Changes

To further evaluate the protective effect of H_2-loaded eye drops, we observed histopathological and morphometrical changes 7 days after retinal I/R injury. First, eye drops with and without H_2 were applied during the entire 90 min process (Duration F in Fig. 4A). Histopathological changes of the retina at 7 days after I/R injury are depicted in Fig. 4B. The H_2-loaded eye drop-treated group showed a nearly normal structure with a thicker retina; however, the H_2-free (vehicle) eye drops-treated group exhibited a marked thinning and atrophy of the retina. Quantitative morphometry of retinal thickness was used to estimate the effect of H_2 (Fig. 4C). The thickness in the I/R-injured retina treated with the H_2-loaded eye drops (102.6 ± 3.8 μm) increased significantly compared with the retina treated with the vehicle (66.9 ± 7.8 μm, $P < 0.0001$). In normal retina from untreated animals, the mean thickness of the retina was 117.0 ± 4.5 μm, indicating that the H_2-loaded eye drops improved the recovery of retinal thickness by >70%.

To investigate the effect of different durations of H_2-loaded eye drop administration, we applied H_2-loaded eye drops using three different time courses (Fig. 4A) and observed that the H_2-loaded eye drops only exerted their effect when H_2 was already inside the eyeball at the onset of reperfusion (Fig. 4C). There were no significant differences in retinal thickness between groups treated with H_2-loaded eye drops only after reperfusion (Duration R; 75.4 ± 10.4 μm) and treated with vehicle ($P = 0.06$). However, the retina that was treated with H_2-loaded eye drops only during ischemia (Duration I; 91.6 ± 7.5 μm) was still significantly thicker than that treated with the vehicle ($P < 0.01$). We next administered H_2-loaded eye drops for 10 min before and 30 min after reperfusion (Duration I/R) and observed that the administration schedule was sufficient to suppress the reduction of retinal thickness (Duration I/R; 96.6 ± 4.4 μm; $P < 0.001$ *vs.* vehicle). Furthermore, we applied eye drops diluted to 25%, 50%, and 75% of the normal H_2-loaded eye drops during the entire 90 min process and observed that H_2-loaded eye drops suppressed the reduction of retinal thickness in a dose-dependent manner (Fig. 4D). It is notable that the 25%-diluted H_2-loaded eye drops were still effective.

H_2-Loaded Eye Drops Restrain Glial Activation

Considering the critical role of increasing glial activation in the pathogenic progression of retinal damage, we investigated the immunohistochemical changes of the Iba1[32] and GFAP[33] at 7 days after retinal I/R injury with and without H_2 treatment. Iba1 is specifically expressed by microglia/macrophages.[34] A small number of Iba1-positive cells was observed in normal retinas, whereas an increasing number of Iba1-positive cells was observed in I/R-injured retinas. At that time, H_2-loaded eye drops were observed to inhibit the activation of microglia (Figs. 5A and 5B), indicating that the ongoing neurodegeneration, which activated microglia, was repressed by H_2. Additionally, H_2-loaded eye drops repressed the increase in GFAP immunoreactivity in I/R-injured retinas. The only GFAP-positive cells in normal retina are astrocytes, whereas in the injured retinas Müller cells, the specific glial cells in the retina, react with anti-GFAP antibody across the retinal layers.[33] In vehicle-treated retinas, GFAP was quite prominent in the Müller cells across the retinal layers and was also strongly present in the astrocytes of the nerve fiber layers, when compared with H_2-loaded eye drop-treated retinas (Fig. 5C).

DISCUSSION

H_2-loaded eye drops have a strong protective effect against retinal I/R injury. Previous studies have demonstrated that antioxidants can decrease retinal injury;[6-15] however, because antioxidants are difficult to deliver into the vitreous body by topical administration, they were injected into either the eye or the peritoneal cavity. Thus, easily applicable anti-oxidative reagents without significant side effects are strongly desired. H_2 is an antioxidant that can easily diffuse into the body. We have observed that H_2 diffuses into the organelles, including mitochondria and the nucleus, of cultured cells.[18] These properties prompted us to attempt the administration of H_2-loaded eye drops for retinal diseases. This is the first report that H_2 can immediately penetrate the vitreous body after the

administration of H_2-loaded eye drops, thereby directly reducing a toxic ROS, •OH, which is produced during I/R. This effectively protects the retina from I/R injury.

Although the sources and mechanisms of ROS generation during I/R by transiently raised IOP are not clearly understood, ROS kills neurons in the ganglion cell layer, inner nuclear layer, and outer nuclear layer mainly by apoptosis.[5,9,35] Ophir *et al*. demonstrated that a burst of •OH occurs in the cat retina during the early reperfusion phase (5 min of reperfusion).[14,15] Thus, we assessed •OH after 15 min of reperfusion with HPF fluorescence and found that the accumulation of •OH was reduced by H_2-loaded eye drops in the I/R-injured retina, indicating that H_2 directly reduced •OH and decreased subsequent oxidative stress. Indeed, one day after reperfusion, H_2-loaded eye drops dramatically decreased 4-HNE-, 8-OHdG-, and TUNEL-positive cells indicating that H_2 protected lipids from peroxidation and DNA from oxidation and reduced subsequent retinal cell death (detected as apoptosis) after I/R injury.

Neurodegeneration was obvious at 7 days after retinal I/R injury. Previous studies on retinal damage 7 days after I/R injury have shown that the thinning of the retina was evident both morphologically and morphometrically.[1,26,36,37] In the present study, H_2-loaded eye drops clearly suppressed the thinning of the retina. However, when H_2-loaded eye drops were applied after the onset of reperfusion (Duration R), they did not protect from retinal damage (Fig. 4). As shown in Fig. 1, H_2 concentration in the vitreous body gradually increased after 2 min and reached its maximum level after 15 min. Immediately after H_2-loaded eye drop administration was stopped, the H_2 level gradually decreased and then completely disappeared after 15 min. Thus, H_2 applied after the onset of reperfusion could not reach a level sufficient to inhibit the accumulation of •OH in the early reperfusion phase, whereas H_2 applied before or during reperfusion (Duration I or I/R) had a high enough H_2 level.

Microglia, Müller cells, and most likely astrocytes respond within hours to elevation of IOP in the retina.[38] Heterogeneous population of microglia/macrophages are observed in the normal retina and activated early after I/R injury.[39] Dying neurons are phagocytosed by them. The long duration of ROS production (up to 48 hours after I/R) may be explained partly by the infiltration of microglia/macrophages into the site of inflammation.[9] The presence of GFAP in a glial cell is considered a marker for reactive gliosis, which is not neuroprotective, but rather promotes neurodegeneration.[40] H_2-loaded eye drops reduced the number of reactive glia, indicating that H_2-loaded eye drops during I/R were sufficient to suppress harmful gliosis after I/R injury and recover the thickness of the retina.

In conclusion, this study demonstrates that the topical application of H_2 can be a useful antioxidant to protect against retinal I/R injury by direct H_2 diffusion into the retina. Accordingly, this neuroprotective antioxidant could offer a new therapeutic strategy to the clinical setting to reduce retinal damage in acute glaucoma and acute retinal vascular occlusion.

References

1. Hughes WF. Quantitation of ischemic damage in the rat retina. *Exp Eye Res*. 1991; 53: 573-582.

2. Kuroiwa S, Katai N, Shibuki H, et al. Expression of cell cycle-related genes in dying cells in retinal ischemic injury. *Invest Ophthalmol Vis Sci*. 1998; 39: 610-617.

3. Osborne NN, Casson RJ, Wood JPM, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retina Eye Res*. 2004; 23: 91-147.

4. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med*. 1985; 312: 159-163.

5. Tezel G. Oxidative stress in glaucomatous neurodegeneration: Mechanisms and consequences. *Prog Retinal Eye Res*. 2006; 25: 490-513.

6. Szabo ME, Droy-Lefaix MT, Doly M, Carré C, Braquet P. Ischemia and reperfusion-induced histologic changes in the rat retina. *Invest Ophthalomol Vis Sci*. 1991; 32: 1471-1478.

7. Muller A, Pietri S, Villain M, Frejaville C, Bonne C, Culcasi M. Free radicals in rabbit retina under ocular hyperpressure and functional consequences. *Exp Eye Res*. 1997; 64: 637-643.

8. Shibuki H, Katai N, Kuroiwa S, Kurokawa T, Yodoi J, Yoshimura N. Protective effect of adult T-cell leukemia-derived factor on retinal ischemia-reperfusion injury in the rat. *Invest Ophthalmol Vis Sci*. 1998; 39: 1470-1477.

9. Shibuki H, Katai N, Yodoi J, Uchida K, Yoshimura N. Lipid peroxidation and peroxynitrite in retinal ischemia-reperfusion injury. *Invest Ophthalmol Vis Sci*. 2000; 41: 3607-3614.

10. Hirooka K, Miyamoto O, Jinming P, et al. Nueroprotective effects of $_D$-allose against retinal ischemia-reperfusion injury. *Invest Ophthalmol Vis Sci*. 2006; 47: 1653-1657.

11. Zhang B, Safa R, Rusciano D, Osborne NN. Epigallocatechin gallate, an active ingredient from green tea, attenuates damaging influences to the retina caused by ischemia/reperfusion. *Brain Res*. 2007; 1159: 40-53.
12. Peachey NS, Green DJ, Ripps H. Ocular ischemia and the effects of allopurinol on functional recovery in the retina of the arterially perfused cat eye. *Invest Ophthalomol Vis Sci*. 1993; 34: 58-65.
13. Li S, Fu Z, Ma H, et al. Effect of lutein on retinal neurons and oxidative stress in a model of acute retinal ischemia/reperfusion. *Invest Ophthalmol Vis Sci*. 2009; 50: 836-843.
14. Ophir A, Berenshtein E, Kitrossky N, et al. Hydroxyl radical generation in the cat retina during reperfusion following ischemia. *Exp Eye Res*. 1993; 57: 351-357.
15. Ophir A, Berenshtein E, Kitrossky N, Averbukh E. Protection of the transiently ischemic cat retina by zinc-desferrioxamine. *Invest Ophthalmol Vis Sci*. 1994; 35: 1212-1222.
16. Song Y, Gong Y, Xie Z, Li C, Gu Q, Wu X. Edaravone (MCI-186), a free radical scavenger, attenuates retinal ischemia/reperfusion injury in rats. *Acta Pharmacol Sin*. 2008; 29: 823-828.
17. Nakao A, Sugimoto R, Billiar TR, McCurry KR. Therapeutic antioxidant medical gas. *J Clin Biochem Nutr.* 2009; 44: 1-13.
18. Ohsawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med*. 2007; 13: 688-694.
19. Cai J, Kang Z, Liu K, et al. Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. *Brain Res*. 2009; 1256: 129-137.
20. Hayashida K, Sano M, Ohsawa I, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun*. 2008; 373: 30-35.
21. Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. *Biochem Biophys Res Commun*. 2007; 361: 670-674.
22. Buchholz BM, Kaczorowski DJ, Suquimoto R, et al. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. *Am J Transplant*. 2008; 8: 2015-2024.
23. Nagata K, Nakashima-Kamimura N, Mikami T, Ohsawa I, Ohta S. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. *Neuropsychopharmacology* 2009; 34: 501-508.
24. Fu Y, Ito M, Fujita Y, et al. Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease. *Neurosci Lett*. 2009; 453: 81-85.
25. Kajiyama S, Hasegawa G, Asano M, et al. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutr Res*. 2008; 28: 137-143.
26. Lam TT. The effect of 3-aminobenzamide, an inhibitor of poly-ADP-ribose polymerase, on ischemia/reperfusion damage in rat retina. *Res Commun Mol Pathol Pharmacol*. 1997; 95: 241-252.
27. Tomizawa S, Imai H, Tsukada S, et al. The detection and quantification of highly reactive oxygen species using the novel HPF fluorescence probe in a rat model of focal cerebral ischemia. *Neurosci Res*. 2005; 53: 304-313.
28. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J Biol Chem*. 2003; 278: 3170-3175.
29. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*. 1992; 119: 493-501.
30. Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med*. 2004; 37: 937-945.
31. Kamiya H. Mutagenicities of 8-hydroxyguanine and 2-hydroxyadenine produced by reactive oxygen species. *Biol Pharm Bull*. 2004; 27: 475-479.
32. Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. A novel gene iba 1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun*. 1996; 224: 855-862.
33. Block F, Grommes C, Kosinski C, Schmidt W, Schwarz M. Retinal ischemia induced by the intraluminal structure method in rats. *Neurosci Lett*. 1997; 232: 45-48.
34. Sasaki Y, Ohsawa K, Kanazawa H et al. Iba1 is an actin-cross-linking protein in macrophages/microglia. *Biochem Biophys ResCommun*. 2001; 286: 292-297.
35. Bonne C, Muller A, Villain M. Free radicals in retinal ischemia. *Gen Pharmac*. 1998; 30: 275-280.
36. Takahashi K, Lam TT, Edward DP, Buchi ER, Tso MOM. Protective effects of flunarizine on ischemic injury in the rat retina. *Arch Ophthalmol*. 1992; 110: 862-870.
37. Junk AK, Mammis A, Savitz SI, et al. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 2002; 99: 10659-10664.
38. Wang X, Tay SS, Ng YK. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. *Exp Brain Res*. 2000; 132: 476-484.
39. Zhang C, Lam TT, Tso MO. Heterogeneous populations of microglia/macrophages in the retina and their activation after retinal ischemia and reperfusion injury. *Exp Eye Res*. 2005; 81: 700-709.
40. Kim K, Ju W, Neufeld AH. Neuronal susceptibility to damage: comparison of the retinas of young, old and old/caloric restricted rats before and after transient ischemia. *Neurobiol Aging* 2004; 25: 491-500.

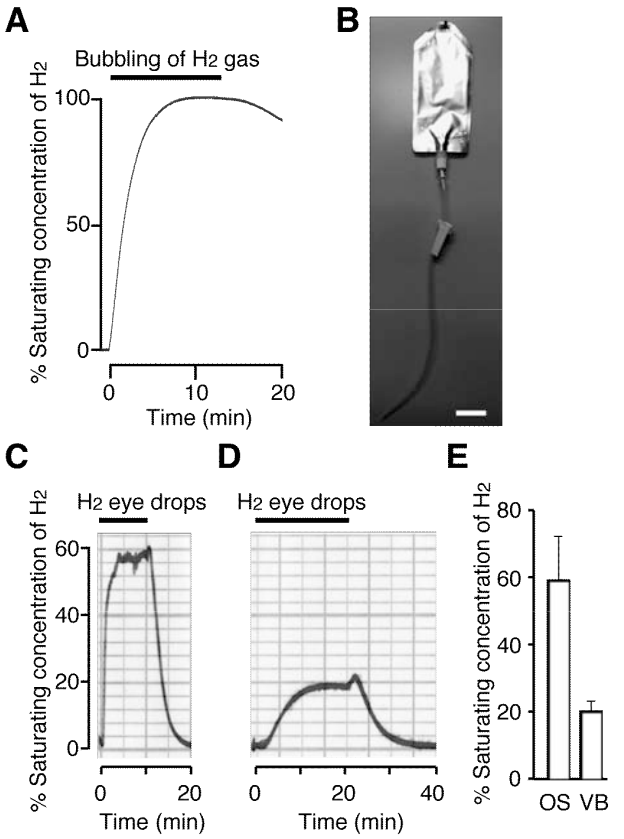

FIGURE 1. H_2-loaded eye drops increase intravitreal H_2. (**A**) H_2-loaded eye drops were prepared by bubbling H_2 gas (shown by a solid thick line, flow rate: 1 l/min) through a 400 ml of normal saline solution. Note that after stop of bubbling, H_2 concentration gradually decreased by stirring and reached <1% within 90 min. (**B**) H_2-loaded eye drops were stored in an aluminum foil bag and administered to the ocular surface with a dropper. Scale bar, 4 cm. The concentrations of H_2 on the ocular surface (**C**) and in the vitreous body (**D**) were monitored with a needle-type H_2 sensor. Periods of H_2-loaded eye drops (67 µl of eye drop per sec) are shown by a solid thick line. (**E**) Summary data showing H_2 concentration on the ocular surface (OS; n = 3) and in the vitreous body (VB; n = 3). Data represent means ± s.d.

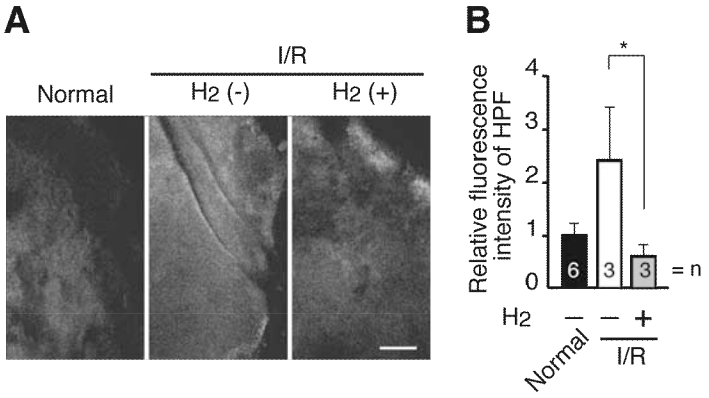

FIGURE 2. H_2-loaded eye drops reduce hydroxyl radicals in the retina. HPF was given intravitreally just before the induction of ischemia. After I/R, retinas were quickly removed and flat mounted. (**A**) Representative fluorescent images were obtained with a laser-scanning confocal microscope. Scale bars, 200 µm. (**B**) HPF fluorescence was quantified from the entire retina of each independent experiment. *P < 0.01. Data represent means ± s.d.

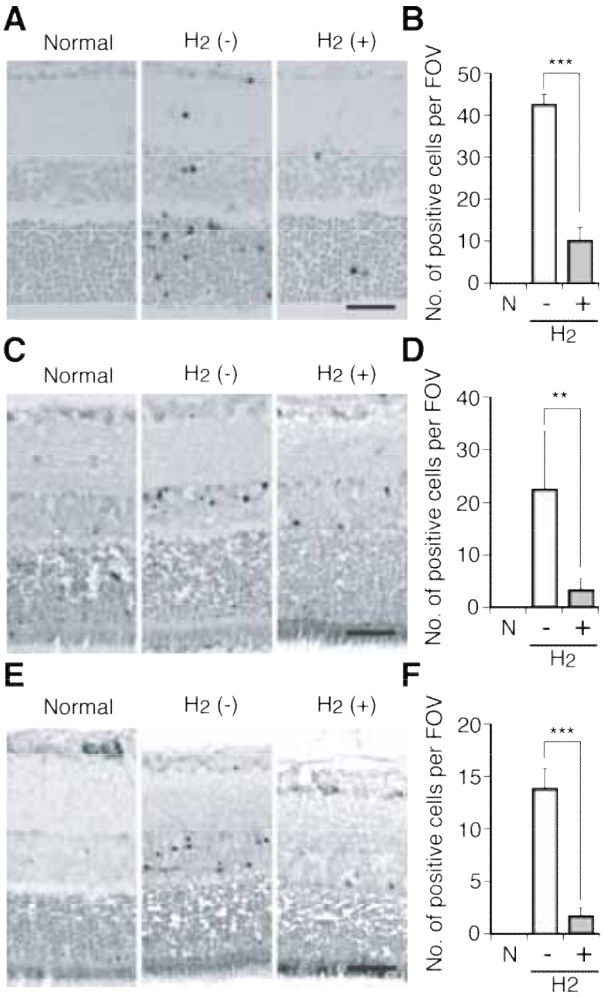

FIGURE 3. H_2-loaded eye drops reduce apoptotic cell death and oxidative stress. One day after I/R, the eyes were immediately enucleated and fixed for the TUNEL assay (**A**, **B**) and staining with antibodies against oxidative stress markers 4-HNE (**C**, **D**) and 8-OHdG (**E**, **F**). Representative slices (**A**, **C**, **E**) and the number of positive cells per field of view (FOV) (**B**, **D**, **F**) in normal retina (N), I/R-injured retinas treated with the vehicle (H_2 (-)), and the H_2-loaded eye drops (H2 (+)) are shown (n = 5 animals per group). Scale bars, 30 μm. $^{**}P < 0.001$. $^{***}P < 0.0001$. Data represent means ± s.d.

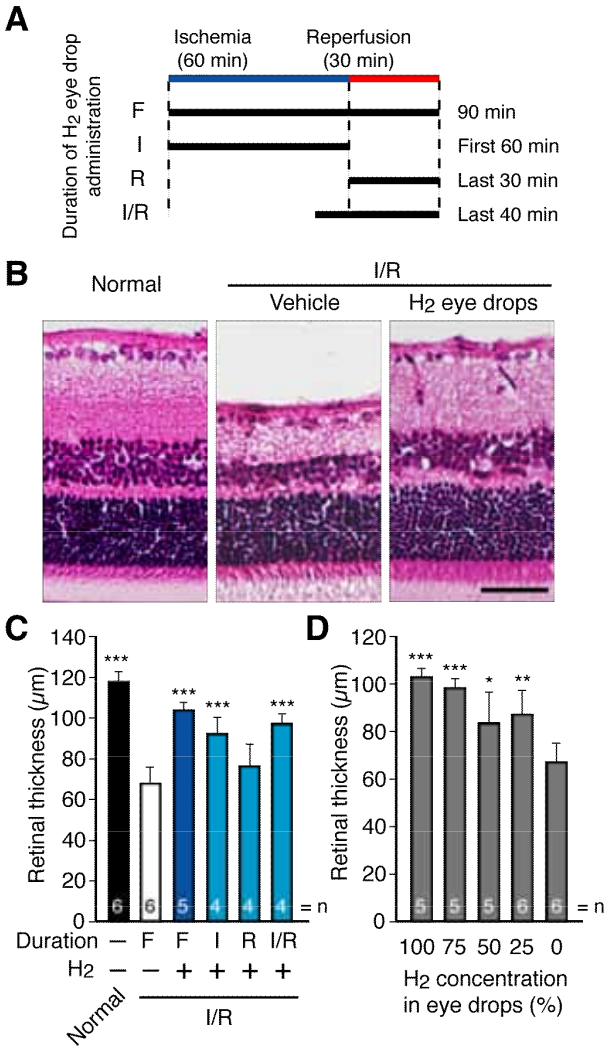

FIGURE 4. H_2-loaded eye drops prevent retinal degeneration caused by I/R. One week after I/R injury, retinas were sliced and stained with H&E. (**A**) Schematic of the experiment, with four different durations of H_2-loaded eye drops administration. (**B**) Representative slices in normal retinas, I/R-injured retinas treated with the vehicle, and retinas treated with H_2-loaded eye drops during the 90 min entire process (60 min of ischemia followed by 30 min of reperfusion) are shown. Scale bar, 50 μm. (**C**) Retinal thicknesses for different durations of H_2-loaded eye drops (100%) administration. $^{***}P < 0.0001$ compared with I/R-injured retina treated with the vehicle (H2 (-)). (**D**) Retinal thicknesses for different concentrations of H_2 in eye drops. Retinas were treated with H_2-loaded eye drops during the entire process (Duration F). $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$ compared with I/R-injured retina treated with 0% H_2. Histograms represent means ± s.d.

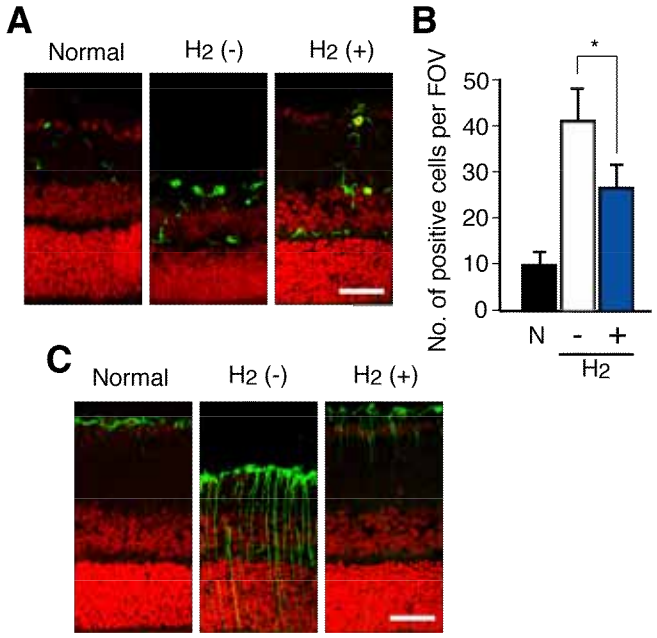

FIGURE 5. H_2-loaded eye drops prevent glial activation caused by I/R in the retina. One-week after I/R injury, retinas were sliced, stained with antibodies to Iba1 (a microglial marker, green) (**A**, **B**) or GFAP (a marker for Müller cells and astrocytes, green) (**C**) and further counterstained for nuclei with propidium iodide (red). Representative slices (**A**, **C**) and the number of positive cells per field of view (FOV) (**B**) in normal retina (N), I/R-injured retinas treated with the vehicle (H2 (-)) and H_2-loaded eye drops (H2 (+)) are shown (n = 5 animals per group). Scale bars, 50 μm. **P* < 0.01. Data represent means ± s.d.