



## Hyperbaric Oxygen Therapy Ameliorates the Blood-Retinal Barrier Breakdown in Diabetic Retinopathy

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**Background:** To study the effect of hyperbaric oxygen (HBO) therapy on diabetic retinopathy in a streptozotocin-induced diabetic rat model. **Methods:** Sprague-Dawley albino male rats were divided into three groups. The three groups were as follow: (1) nondiabetic control group (non-DM control), (2) diabetic control group (DM control), and (3) diabetic rats receiving hyperbaric oxygen therapy (DM HBO). Rats in DM HBO group were incubated in an oxygen monoplace chamber. The hyperbaric oxygen condition was set at 2.5 atmospheres and 100% oxygen. The duration of a single HBO treatment was 90 minutes. Rats in DM HBO groups received HBO 3 times per week for 3 months. Retinal vascular permeability was assessed by measuring FITC-labeled bovine albumin and retinal Evans blue leakage into the retina. **Results:** We found that the retinal parenchyma showed prominent thickening but not statistically significant in rats with DM, corresponding to the retinal edema, compared with the control and DM HBO groups. FITC relative fluorescence intensity (Mean  $\pm$  SE) in normal control animals, diabetic animals, and HBO-treated diabetic animals was  $356 \pm 47$ ,  $865 \pm 78$ , and  $518 \pm 49$ , respectively, demonstrating significant difference between the means of diabetic and HBO-treated diabetic animals, and between means of control and diabetic animals ( $n=8$ ,  $P<0.05$ ). Retinal Evans blue leakage in control animals, diabetic animals, and HBO-treated diabetic animals was  $7.6 \pm 2.9$ ,  $18.5 \pm 4.2$  and  $10.2 \pm 3.1 \mu\text{l plasma} \times \text{g retinal dry weight}^{-1} \text{h}^{-1}$ , respectively, demonstrating significant difference between the means of diabetic and HBO-treated diabetic animals, and between means of control and diabetic animals ( $n=8$ ,  $P<0.05$ ). **Conclusions:** HBO therapy may diminish the extent of the increased blood-retinal barrier breakdown in diabetic animals.

Key words: blood-retinal barrier, diabetic retinopathy, hyperbaric oxygen therapy

### INTRODUCTION

Diabetic retinopathy is the most common cause of blindness in the working population of the developed countries and also a significant cause of blindness in the elderly. Among the diabetics the overall prevalence of retinopathy is approximately 26%. Diabetic retinopathy has been known to result from microangiopathy caused by diabetes. However, the pathogenesis of microangiopathy is still poorly understood<sup>1</sup>.

The single greatest source of vision loss in diabetes is macular edema<sup>2</sup>; pathology is a direct consequence of

diabetic blood-retinal barrier (BRB) breakdown. Breakdown of the BRB characterizes early stages of vascular dysfunction in both human and experimental diabetes<sup>3,4</sup>. The mechanism of BRB breakdown has been thought due to defects in the inner BRB, including opening of interendothelial cell tight junctions<sup>5</sup>, endothelial cell transcytosis<sup>6</sup>, and vacuolation of the retinal pigment epithelium<sup>7</sup>.

Based on the conclusions from Diabetic Retinopathy Study which is a multicenter clinical trial focusing on the effects and efficacy of photocoagulation on diabetic retinopathy, panretinal photocoagulation is indicated for any eye with Diabetic Retinopathy Study high-risk characteristics, rubeosis iridis, or neovascular glaucoma. Macular photocoagulation is indicated for clinically significant macular edema. Although focal laser photocoagulation is effective at reducing the risk of moderate visual loss by approximately 50%, significant numbers of patients continue to lose vision<sup>8</sup>. Focal laser treatment results in permanent paracentral scotomas, mild visual field defects,

Received: May 22, 2003; Revised: October 22, 2003; Accepted: October 27, 2003.

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and addresses only late complications of diabetes. Currently no preventive treatment for the ocular complications of diabetes mellitus exists, especially an effective pharmacological treatment for diabetic macular edema.

Hyperbaric oxygen (HBO) therapy is defined as treatment in which a patient breathes 100% oxygen while under a pressure greater than 1 atmosphere. The efficacy of hyperbaric therapy is based on a reduction in volume of gas-filled spaces; and an elevation of the partial pressure of oxygen resulting in hyperoxygenation of perfused tissues. Beneficial HBO effects have been claimed for gas embolism, decompression sickness, blood loss, anemia, carbon monoxide poisoning, gas gangrene, comprised skin flaps and grafts, acute traumatic and/or occlusive/embolic ischemic stroke, and problem wounds<sup>9</sup>.

Some potent vasopermeability factors including vascular endothelial growth factor (VEGF) may be operative in the pathogenesis of diabetic blood-retinal barrier breakdown<sup>10,11</sup>. Retinal VEGF levels are upregulated in diabetes and coincide with blood-retinal barrier breakdown in humans and rats<sup>12-14</sup>. Hyperoxia causes decreased expression of VEGF in adult retina<sup>15</sup>. HBO therapy is able to lead an elevation of the partial pressure of oxygen in perfused tissues and therefore, possibly restores an adequate oxygen tension in ischemic retina in diabetes mellitus. In the present study, we sought to determine the effect of HBO on blood-retinal barrier breakdown in diabetic retinopathy.

## MATERIALS AND METHODS

Male Sprague-Dawley albino male rats, weighting approximately 200 g, were used in this study. The animals were treated for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In all procedures involving diabetic animals, rats were fasted for a 24-hour period.

Rats were divided into three groups, with 12 rats in each group. The three groups were as follow: (1) nondiabetic control group (non-DM control), (2) diabetic control group (DM control), and (3) diabetic rats receiving hyperbaric oxygen therapy (DM HBO).

### Induction and Maintenance of Diabetes Mellitus with Streptozotocin

Under intraperitoneal or intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia, diabetic rats in the DM control and DM HBO groups were then induced with a single 65 mg/kg intraperitoneal injection of streptozotocin in 1 mM citrate buffer, pH 4.5. Animals that served as nondiabetic controls received an equivalent

amount of citrate buffer alone. Twenty-four hours later, rats with blood glucose levels greater than 250 mg/dl were declared diabetic. Exactly 1 week later, just before experimentation, blood glucose levels were assayed again to confirm diabetic status. Blood glucose levels were measured every other week until they were sacrificed. Diabetic animals received 2 to 3 units of NPH insulin weekly to prevent ketosis. General conditions of all rats were monitored during the study.

### Hyperbaric Oxygen Therapy (HBO)

Rats in DM HBO groups were incubated in an oxygen monoplace chamber. The hyperbaric oxygen condition was set at 2.5 atmospheres 100% oxygen. The duration of single HBO treatment was 90 minutes.

Rats in DM HBO groups started to receive HBO every Monday, Wednesday, and Friday once rats were claimed to be diabetic. The experiment regimen lasted for 3 months. At the end of the experiment, rats were sacrificed and eyes were harvested for further process.

### Measurement of Blood-Retinal Barrier Using FITC-Labeled BSA

BRB permeability was measured in experiment using a modification of the method described by Enea et al<sup>4</sup>. Under ketamine/xylazine anesthesia (80/0.8 mg/kg i.p.), rats received tail vein injection of 100 mg/kg of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) (Sigma). After 20 minutes, the animals were sacrificed, and the eyes were enucleated. One eye was immediately placed in 10% phosphate-buffered formalin for 48 hours followed by paraffin embedding. At the time of death, blood was collected into EDTA-containing tubes that were centrifuged at 2,000 g for 10 minutes. Plasma was assayed for fluorescence with a fluorescence spectrophotometer based on standard curves of FITC-BSA in normal rat plasma with excitation at 433 nm and emission at 455 nm. FITC is covalently bound to BSA.

More than 20 serial 6- $\mu$ m paraffin-embedded axial section were obtained starting at optic disc. After staining with hematoxylin and eosin (H & E) reagent, 10 intact sections of equal length, each 30  $\mu$ m apart, were evaluated. Eyes with retinal detachment were excluded from this experiment. The sections were viewed with an Olympus OM-2 fluorescence microscope fitted with a Sony CLD video camera. Fluorescence intensities of digital images of nonvascular segments of retina in the middle portion between optic disc and ora serrata were measured using Optimas software on an IBM PC. Fluorescence intensities of 12 nonvascular retinal points were collected from each

section of control, DM control, and DM HBO groups. The average retinal fluorescence intensity was then normalized to a noninjected control retina and to plasma fluorescence intensity of each animal. Through serial sectioning of each eye, this technique enabled quantification of increased vascular permeability in the retina.

### Measurement of Blood-Retinal Barrier Breakdown Using Evans Blue

The change of BRB permeability in diabetic retina was further confirmed in experiment using a modification of the method described by Xu Q et al<sup>16</sup>. Under ketamine/xylazine anesthesia (80/0.8 mg/kg i.p.), the right femoral vein and right iliac artery were cannulated with polyethylene tubing, and filled with heparinized saline (250 units heparin/ml saline). Evans blue was injected through the femoral vein at a dosage of 50 mg/kg. Two minutes later, 0.2 ml blood was drawn from the iliac artery to obtain the initial Evans blue plasma concentration. At 15-minute intervals, 0.1 ml blood was drawn from right iliac artery up to 2 hours after injection of Evans blue to obtain the time-averaged Evans blue plasma concentration. At 2 hours after injection, the chest cavity was opened and 0.2 ml blood was drawn from the ventricle to obtain the final Evans blue plasma concentration. Subsequently rat heart was perfused for 2 minutes at 37°C with citrate buffer (0.05 M, pH 3.5). After perfusion, both eyes were enucleated and retinas were carefully dissected away. After measurement of the retinal wet weight, retinas were dried in a Speed-Vac. The Evans blue was extracted by incubating every single retina in 120  $\mu$ l formamide for 18 hours at 70°C. The extract was centrifuged at a speed of 70,000 rpm for 1 hour at a temperature of 4°C. 60  $\mu$ l of the supernatant was used for triplicate spectrophotometric measurements. A background-subtracted absorbance was determined by measuring each sample at 620 nm, the absorbance maximum for Evans blue, and 740 nm, the absorbance minimum. The concentration of Evans blue in each extract was determined from a standard curve of dye in formamide. Blood-retinal barrier breakdown was calculated and expressed in  $\mu$ l plasma  $\times$  g retinal dry weight<sup>-1</sup>h<sup>-1</sup>.

### Statistics

Normally distributed data in two groups were analyzed with a Student's t-test. All multiple comparisons used an analysis of variance (ANOVA). Differences were considered statistically significant if  $P < 0.05$ . All numerical results were expressed as means  $\pm$  SE.

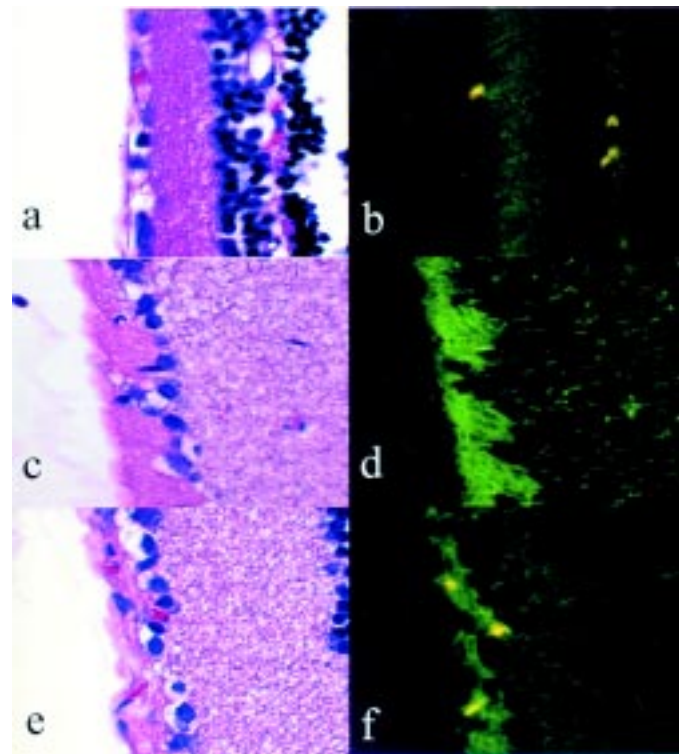


Fig.1 Fluorescence micrographs of hematoxylin & eosin-stained retinal sections (a,b) from nondiabetic control animals, (c,d) from diabetic control animals, and (e,f) from HBO-treated diabetic animals. Retinal parenchymal fluorescence intensities increased in diabetic control animals compared with nondiabetic control animals. HBO effectively suppresses FITC-labeled BSA leakage from retinal vessels in HBO-treated diabetic animals. ( $\times 200$ )

## RESULTS

### Induction of Diabetes

The blood glucose levels in non-diabetic rats and diabetic rats, just before experimentation, were  $87 \pm 4$  mg/dl (range, 73-97 mg/dl) and  $383 \pm 27$  mg/dl (range, 295-460 mg/dl), respectively ( $P < 0.0001$ , student's t-test). There were no changes in diabetic status compared with the earlier days postinduction of diabetes. That meant all diabetic rats remained diabetic during the whole experimental period.

### Histopathologic Examination Using Hematoxylin & Eosin Staining and Fluorescence Microscopy

Although not statistically significant, light-microscopic examination using H & E-stained retinal sections (Fig. 1) revealed prominent retinal parenchyma thickening, corresponding to the retinal edema, in DM control animals

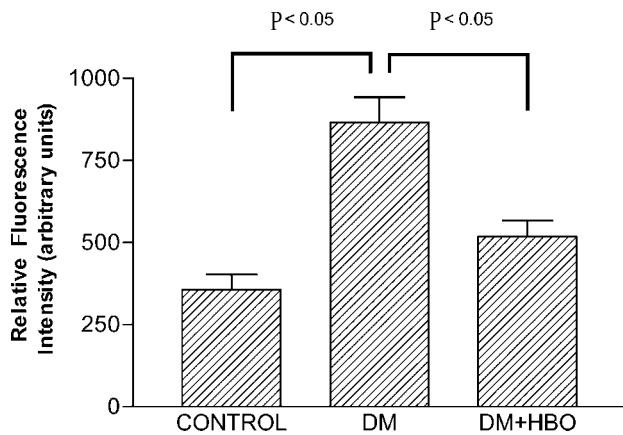


Fig.2 Hyperbaric oxygen therapy suppresses blood-retinal barrier breakdown. The average relative fluorescence intensities in normal nondiabetic control, diabetic control, and HBO-treated diabetic rats.

compared with non-DM control animals and DM HBO animals. The thickened retinal parenchyma in DM control animals was largely due to the increased thickness of the nerve fiber layer and inner plexiform layer. Prominent fluorescence was seen in nerve fiber layer and inner plexiform layer in DM control animals compared with non-DM control animals and DM HBO animals. Retinal parenchymal fluorescence intensity seemed to decrease after HBO treatment in DM HBO animals compared with DM control animals.

#### Semiquantitation of Blood-Retinal Barrier Breakdown Using FITC-Labeled BSA

Figure 2 shows that FITC relative fluorescence intensity (Mean  $\pm$  SE) in non-DM control animals, DM control animals and DM HBO animals was  $356 \pm 47$ ,  $865 \pm 78$ , and  $518 \pm 49$ , respectively, demonstrating significant difference between the means of diabetic and HBO-treated diabetic animals, and between means of control and diabetic animals ( $n=8$ ,  $P<0.05$ ).

#### Quantitation of Blood-Retinal Barrier Breakdown Using Evans Blue

Figure 3 demonstrates that Evans blue standards in formamide maintain a reliable linear relationship between background-subtracted absorbance (620-740 nm) and concentration from 50 to 1,000 ng/ml.

Figure 4 shows that the retinal vascular leakage (permeability) of Evans blue (mean  $\pm$  SE) in non-DM control animals, DM control animals and DM HBO animals was  $7.6 \pm 2.9$  ( $n=10$  retinas),  $18.5 \pm 4.2$  ( $n=9$  retinas)

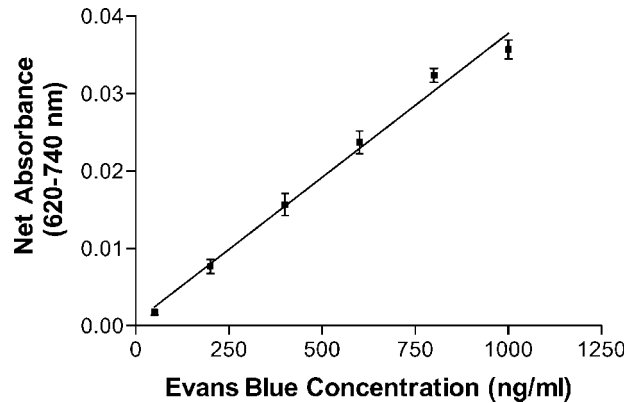


Fig.3 A standard curve for Evans blue in formamide. Background-subtracted absorbance (620-740 nm) of Evans blue standards in formamide as a function of concentration from 50 to 1,000 ng/ml.

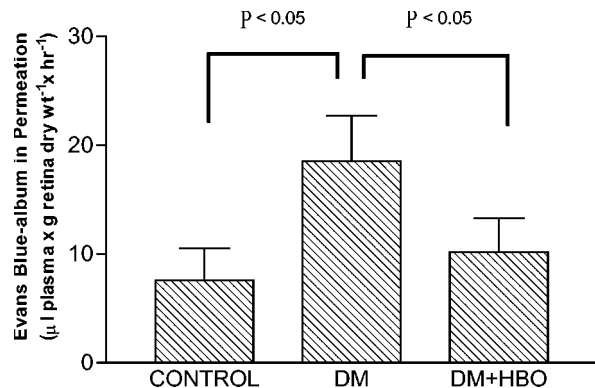


Fig. 4 Hyperbaric oxygen therapy suppresses blood-retinal barrier breakdown. Retinal Evans blue levels in normal nondiabetic control, diabetic control, and HBO-treated diabetic rats.

and  $10.2 \pm 3.1$  ( $n=10$  retinas)  $\mu\text{l plasma} \times \text{g retinal dry weight}^{-1} \times \text{hr}^{-1}$ , respectively, demonstrating significant difference between the means of diabetic and HBO-treated diabetic animals, and between means of control and diabetic animals. ( $P<0.05$ )

#### DISCUSSION

This study investigated the effect of long-term hyperbaric oxygen therapy on the BRB breakdown occurring in an animal model of diabetic retinopathy. We also examined the histopathologic changes of retinal sections and quantitatively assessed the effect of HBO on BRB breakdown by FITC-labeled BSA and Evans blue.

We assessed BRB breakdown in two established ways: indirect measurement of BRB permeability using FITC-labeled BSA and direct measurement of BRB permeability using Evans blue. Markedly increased retinal vascular permeability was observed in diabetic control animals compared with nondiabetic control animals and diabetic animals receiving HBO. Furthermore, HBO significantly lowered retinal vascular permeability in diabetic control animals to near-control levels.

Leuenberger et al.<sup>17</sup> has described the histopathologic changes of the streptozotocin rats. Streptozotocin-induced diabetic rats developed cataract, neovascularization of iris, loss of retinal capillary endothelial and mural cells, focal basement membrane thickening, and variations in capillary diameter, resulting in the appearance of fusiform microaneurysms. Several researchers have described the breakdown of the BRB occurring in the streptozotocin rats<sup>12,13,18-20</sup>. Ishibashi et al.<sup>5</sup> noted that the increased permeability in retinal capillaries of streptozotocin rats preceded the thickening of the basement membrane and seemed to play an important role in the development of diabetic retinopathy.

The major detrimental effect caused by the BRB breakdown in human is macular edema. Diabetic macular edema is caused by either focal or diffuse leakage<sup>21</sup>. Intraretinal edema results from leakage of fluid through dysfunctional retinal endothelial cells. Focal retinal thickening is nearly always caused by leaking microaneurysms. Diffuse retinal thickening is caused a generalized breakdown of the inner BRB.

The critical role of VEGF in the development of diabetic retinopathy has been proposed<sup>22</sup>. VEGF, a potent vasopermeability factor, has shown to be operative in the pathogenesis of diabetic BRB breakdown<sup>10,11</sup>. Mathews et al. used histochemical analysis to demonstrate that increased VEGF immunoreactivity in diabetic retinal vessels is related to increased vascular permeability, as indicated by human serum albumin immunostaining<sup>22</sup>. In the same experiment, he further demonstrated that VEGF appears to be increased in diabetic subjects before the onset of retinopathy. More observations also pointed out that retinal VEGF levels are upregulated in diabetes and coincide with BRB breakdown in humans and rats<sup>12-14</sup>.

HBO is a treatment in which a patient breathes 100% oxygen while under a pressure greater than 1 atmosphere. The efficacy of HBO is based on a reduction in volume of gas-filled spaces and an elevation of the partial pressure of oxygen resulting in hyperoxygenation of perfused tissues. Beneficial HBO effects have been claimed for gas embolism, decompression sickness, blood loss, anemia, car-

bon monoxide poisoning, gas gangrene, comprised skin flaps and grafts, acute traumatic and/or occlusive/embolic ischemic stroke, and problem wounds<sup>9</sup>. HBO is effectively used for problem wounds, especially diabetic foot infections and leg ulcers caused by arterial insufficiency. In such a way DM foot infection and ulceration healed at a faster rate compared to the traditional management. Recently HBO has been applied in several ocular diseases especially in central/branch retinal artery occlusion, and central/branch retinal vein occlusion<sup>23,24</sup>. Roy et al.<sup>24</sup> has described that HBO can lead to reduction of vascular permeability and effectively decrease the cystoid macular edema.

Our present study provides additional preclinical evidence that HBO may be a useful alternative method in the prevention and treatment of ocular complications of diabetes mellitus, especially in persistent macular edema due to BRB breakdown in diabetic retina. VEGF has been shown to be related to the pathogenesis of diabetic blood-retinal barrier breakdown<sup>25,26</sup>. Some data have shown retinal VEGF levels are upregulated in diabetes and coincide with blood-retinal barrier breakdown in humans and rats<sup>12-14</sup>. Hyperoxia causes decreased expression of VEGF in adult retina<sup>15</sup>. HBO therapy is able to lead an elevation of the partial pressure of oxygen in perfused tissues. Taken together, we believe that HBO, by restoring an adequate oxygen tension in ischemic retina in diabetes mellitus, leads to downregulation of VEGF expression with subsequent amelioration of retinal vascular leakage.

One major concern for the usage of HBO is the possible oxygen toxicity in retina caused by hyperoxic condition. Previous studies have shown that exposure of neonatal animals to hyperoxia results in obliteration of retinal vessels<sup>27</sup>, while adult retinal vessels are resistant to damage<sup>28</sup>. For instance, inhalation of 70% oxygen for 48 hours results in mild reversible constriction of retinal vessels in an adult animal, while in a neonate with an immature retina there is permanent occlusion of many retinal vessels<sup>29</sup>. Retinal vascular development is stimulated by increased VEGF expression in avascular peripheral retina, which is decreased when vascularization relieves the physiologic hypoxia<sup>30</sup>. Hyperoxia results in premature downregulation of VEGF expression in the retina and obliteration of newly formed vessels. Therefore VEGF is a survival factor for newly formed retinal vessels. Yamada et al have demonstrated that the decreased susceptibility of mature vs. immature retinal vessels to the damaging effects of hyperoxia<sup>15</sup>, which is good for regression and amelioration of newly formed vessels in proliferative diabetic retinopathy.

Our data indicate that chronic HBO can significantly reduce retinal vascular leakage in an animal model of diabetic retinopathy. The clinical development of a safe and effective regime for the prevention of the leading cause of vision loss in diabetes, macular edema, would have a major public health impact.

## ACKNOWLEDGEMENTS

This study was supported by grants DOD-92-48 from the Ministry of National Defense and TSGH-C92-33 from the Tri-Service General Hospital.

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