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A Method for Chorioretinal Oxygen Tension Measurement

Mahnaz Shahidi,

Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

Akbar Shakoor,

Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

Norman P. Blair,

Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

Marek Mori, and

Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

Ross D. Shonat

Department of Biomedical Engineering, Worcester Polytechnic Institute Worcester, Massachusetts, USA

Abstract

Purpose—To report an optical imaging system that was developed to measure oxygen tension (pO_2) in the chorioretinal vasculatures. The feasibility of the system for the measurement of changes in pO_2 separately in the retinal and choroidal vasculatures was established in rat eyes by varying the fraction of inspired oxygen and inhibiting nitric oxide activity.

Methods—Our optical section phosphorescence imaging system was modified to provide quantitative measurements of pO_2 separately in the retinal and choroidal vasculatures. A narrow laser line was projected at an angle on the retina after intravenous injection of an oxygen-sensitive probe (Pd-porphyrin), and phosphorescence emission was imaged. A frequency-domain approach allowed measurements of the phosphorescence lifetime by varying the phase relationship between the modulated excitation laser light and sensitivity of the imaging camera. Chorioretinal pO_2 was measured while varying the fraction of inspired oxygen and during intravenous infusion of N^G -nitro-L-arginine (N^G -NLA), a nonselective nitric oxide synthase inhibitor.

Results—The systemic arterial pO_2 varied according to the fraction of inspired oxygen. The pO_2 in the retinal and choroidal vasculatures increased as the fraction of inspired oxygen was increased. Compared with base-line, choroidal pO_2 decreased during infusion of N^G -NLA, whereas the pO_2 in the retinal vasculatures remained relatively unchanged. The choroidal pO_2 decreased markedly with each incremental increase in N^G -NLA infusion rate, in the range 1–6 mg/min, and there was no additional change in the choroidal pO_2 at N^G -NLA infusion rates above 6 mg/min.

Conclusions—An optical method combining pO_2 phosphorescence imaging with chorioretinal optical sectioning was established that can potentially be applied for better understanding of retinal and choroidal oxygen dynamics in physiologic and pathologic states.

Keywords

Choroid; imaging; oxygen; retina

INTRODUCTION

The availability of oxygen and other metabolites is necessary for maintaining the normal function of the retinal tissue. Retinal oxygenation has been shown to be compromised in acute retinal artery embolic disease and retinal vein occlusion.^{1,2} Additionally, inadequate retinal oxygenation has been implicated in the development of common eye diseases such as diabetic retinopathy, glaucoma, and age-related macular degeneration.^{3–7} Because oxygenation of the retinal tissue relies on both the retinal and choroidal circulations, determining the relative contributions of these vasculatures to oxygenation of the retinal tissue may be useful in furthering the understanding of retinal physiology in health and disease.

Several techniques are currently available for studying retinal oxygenation. Multiwavelength reflectance spectrophotometry^{8,9} measures oxygen saturation. The relationship between oxygen tension (pO_2) and the oxygen saturation of hemoglobin is determined by the oxygen-hemoglobin dissociation curve and may be altered due to various metabolic conditions. Retinal tissue oxygenation under varying physiological conditions has been studied using oxygen-sensitive microelectrodes,¹⁰ but this method is invasive and thus has the potential to disturb the retinal microenvironment. Magnetic resonance spectroscopy has been used to measure pO_2 of the inner retina.¹¹ Recently, retinal oxygen tension measurement has been performed non-invasively using magnetic resonance imaging¹² but is limited in resolution as compared with optical imaging. Intravascular oxygen tension has been measured using phosphorescence imaging^{13,14} but is limited in depth discrimination. Aside from the intravenous injection of the probe, phosphorescence imaging is a noninvasive technique with a low propensity to alter the retinal microenvironment.

We have previously reported the feasibility of a novel optical imaging system for assessment of oxygenation separately in the choroidal and retinal vasculatures by projecting a laser slit obliquely on the retina and measuring the intensity of phosphorescence from an oxygen-sensitive molecular probe.¹⁵ In the current study, this optical section phosphorescence imaging system was modified to provide quantitative measurements of pO_2 in the chorioretinal vasculatures by determining the phosphorescence lifetime. The feasibility of the system for determining pO_2 changes separately in the retinal and choroidal vasculatures was established by varying the fraction of inspired oxygen in rats. Furthermore, because inhibition of nitric oxide synthase (NOS) has been shown to be associated with decreased blood flow in the choroid and, to a lesser degree, in the retinal vascular bed,^{16–24} this model was also used in the current study to establish the feasibility of our system for measuring chorioretinal pO_2 changes.

MATERIALS AND METHODS

Instrumentation

Our optical section phosphorescence imaging system¹⁵ was modified to measure the phosphorescence lifetime of meso-tetra (4-carboxyphenyl) porphine (Pd-porphyrin), an oxygen-sensitive molecular probe. A frequency-domain approach was used for the measurement of phosphorescence lifetime as previously described.^{13,25–27} In this approach, the light that is used for excitation of the probe and the sensitivity of the camera that is used for detection of the phosphorescence emission are independently modulated. By varying the phase relationship between the two modulators, phosphorescence phase and lifetime are

derived. The phosphorescence lifetime is related to the pO_2 according to the Stern-Volmer expression:

$$\tau_0/\tau = 1 + (\kappa_Q)(\tau_0)(pO_2)$$

where pO_2 (mmHg) is the oxygen tension, τ (μs) is the phosphorescence lifetime, κ_Q (1/mmHg μs) is the quenching constant for the triplet-state phosphorescence probe, and τ_0 is the lifetime in zero oxygen environment.

The schematic diagram of the chorioretinal pO_2 imaging system is shown in Fig. 1. A diode laser (Beta Electronics Inc., Columbus, OH, USA) at a wavelength of 532 nm was expanded and focused on the retina. A neutral-density filter was placed in front of the laser to reduce its power. A spherical and a cylindrical lens were placed in the path of the incident laser beam to form a focused line on the retina. In the imaging path, the optics of the slit-lamp biomicroscope formed an image of the retina onto a digital intensified charge coupled device (ICCD) camera (Roper Scientific, Trenton, NJ, USA). An infrared filter having a cutoff wavelength of 645 nm, with transmission overlapping the phosphorescence emission of the oxygen probe, was placed in the imaging path. Due to the angle (10°) between the incident laser and imaging path, a chorioretinal optical section phosphorescence image was acquired by the digital ICCD camera. The excitation laser beam was modulated by placing a spinning optical chopper (Photon Technology, London, Ontario, Canada) in the beam path. The digital output from the chopper triggered the timing controller of the ICCD camera. The timing of the image acquisition with respect to the trigger signal was controlled by a computer using the software provided by the ICCD camera.

A set of six phase-delayed optical section phosphorescence images was acquired by incrementally delaying the modulated ICCD intensifier with respect to the modulated excitation laser beam, similar to a previously described method.¹³ In the current experimental setup, the optical chopper frequency was set at 1600 Hz (full cycle = 625 μs), the intensifier gate width was set at half cycle or 312.5 μs , and the time delay increment was 63 μs , thereby producing phase shifts between 0° and 180° . In each animal and under each experimental condition, three to five sets of phase-delayed optical section phosphorescence images were acquired.

An example of an optical section phosphorescence image, displaying a cross-sectional view of the retinal and choroidal vasculatures, is shown in Fig. 2A. The phosphorescence from the probe is visualized distinctly in the retinal artery, vein, capillaries, and choroid. The optical section phosphorescence images were analyzed to provide pO_2 measurements in the chorioretinal vasculatures. Three vascular areas corresponding to a retinal artery, vein, and choroid were identified on the optical section phosphorescence image as described previously.¹⁵ A fourth area, adjustable in extent, based on the observer's ability to discern the microvasculatures, was selected corresponding to the retinal capillaries. Retinal veins were identified as highly phosphorescent structures. Retinal arteries were smaller, less phosphorescent structures as compared with the veins and were situated between two retinal veins. Retinal capillaries were visualized as smaller structures situated between a retinal vein and an artery. The choroid was identified as a large band of phosphorescence posterior to the retinal veins and arteries. The average intensity in each of these four vascular areas was determined in the set of phase-delayed images. A previously developed dedicated software algorithm¹³ was used to calculate the phase shift, phosphorescence lifetime, and pO_2 in each of the four selected vasculatures based on the intensity averages derived from the set of phase-delayed images. An example of a set of six phase-delayed images is shown in Fig. 2B. A box was placed on a retinal vein, and the phosphorescence intensity within the box was plotted as

a function of phase delay. Based on the best-fit sinusoidal curve,¹³ a phase shift of 38.2°, corresponding to a pO₂ measurement of 29.4 mmHg, was determined using κ_Q and τ_0 values of 381 mmHg⁻¹ s⁻¹ and 637 μ s, respectively.²⁸ The average pO₂ measurements in the chorioretinal vasculatures and the systemic arterial pO₂ were statistically compared using analysis of variance (ANOVA) and Bonferroni adjustment for pair-wise comparison. Statistical significance was accepted at $p < 0.05$.

The probe calibration has been performed previously.¹³ Instrument phase error was measured and eliminated at hardware before *in vivo* experiments. The systemic instrumentation phase error was established at the beginning of each experiment by measuring the phase shift from backscattered light without any probe. Additionally, the phase shift corresponding to zero pO₂ was determined using the probe solution equilibrated in a 0% oxygen environment. The 0% oxygen environment was generated in a cuvette by adding glucose oxidase (Sigma, St. Louis, MO, USA) to a solution of probe dissolved in a normal saline (100 μ M) containing bovine serum albumin (500 μ M) and β -D-glucose (40 mM) (Sigma). The difference between the measured and calculated phase shift in 0% oxygen environment was used to adjust the measured phase shifts obtained in animal experiments. The dependence of the measured phosphorescence lifetime on flow was also investigated. A probe solution at pO₂ of 30 mm Hg was pumped through a capillary tube and phosphorescence lifetime was measured under flow rates of 0, 20, 40, 100, 200, 400 μ L min. The phosphorescence lifetime measurements under varying flow rates were similar ($p = 0.1$).

Animals

Eleven male Long Evans pigmented rats (450–650 g) were used for this study. The animals were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rats were anesthetized using ketamine (85 mg/kg i.p.) and xylazine (3.5 mg/kg i.p.). Anesthesia was maintained by an intraperitoneal infusion of ketamine and xylazine at the rate of 0.5 mg kg⁻¹ min⁻¹ and 0.02 mg kg⁻¹ min⁻¹, respectively. The pupils were dilated with 2.5% phenylephrine and 1% tropicamide. The Pd-porphyrin probe (Frontier Scientific, Logan, UT, USA) was dissolved (12 mg/ml) in bovine serum albumin solution (60 mg/ml) and physiological saline buffered to a pH of 7.4 and injected intravenously (15 mg/kg) via a tongue or penile vein. Prior to imaging, 1% hydroxypropyl methylcellulose was applied to the cornea, and a glass coverslip was placed on the cornea in order to eliminate its refractive power and to prevent corneal dehydration. Rectal temperature was maintained between 37°C and 38°C via a copper tubing water heater. The rat was placed in front of the imaging instrument. The laser power was adjusted to 100 microwatts, which is safe for viewing according to the American National Standard Institute for Safety Standards.²⁹

The fraction of inspired oxygen was varied in six rats via a high-flow face mask system. Gas mixtures containing 10% oxygen, 50% oxygen, and room air (21% oxygen) were administered to the rats 10 min before and during chorioretinal pO₂ imaging. During administration of the three incremental fractions of inspired oxygen, concurrent with imaging, arterial blood was drawn through the femoral artery catheter and sent for blood gas analysis to provide measurements of systemic arterial pO₂.

Blood flow was altered in five rats by infusion of *N*^ω-nitro-L-arginine (*N*^ω-NLA) (Sigma), which served as a nonselective NOS inhibitor. *N*^ω-NLA was administered at single infusion rates in three rats and at multiple infusion rates in two rats. The rats were anesthetized and premedicated with atropine (1 mg/kg subcutaneously). The left femoral vein was cannulated and the catheter was attached to a 25-cc syringe mounted on a syringe pump. Immediately before infusion, *N*^ω-NLA was dissolved in normal saline (10 mg ml⁻¹) with 4 N NaOH added

to result in a pH of 9.3. Imaging was performed at baseline (prior to infusion of N^{ω} -NLA) and during infusion of N^{ω} -NLA at varying rates, in the range of 1 to 16 mg/min.

RESULTS

The systemic arterial pO_2 was found to vary according to the fraction of inspired oxygen. Under 10%, 21%, and 50% oxygen breathing conditions, the mean systemic arterial pO_2 in six rats was measured to be 27 ± 4 mmHg, 53 ± 7 mmHg, and 139 ± 34 mmHg, respectively. Measurements of chorioretinal pO_2 under 10%, 21%, and 50% oxygen breathing conditions are shown in Fig. 3. Chorioretinal and systemic arterial pO_2 measurements obtained during the three oxygen breathing conditions were significantly different ($p < 0.001$). Significant differences were found in the pO_2 of the vascular beds under 10% ($p = 0.01$), 21% ($p < 0.001$), and 50% ($p < 0.001$) breathing conditions. Under 10% oxygen breathing condition, the mean pO_2 in the retinal vein was significantly lower ($p = 0.004$) than the choroidal pO_2 . Under 21% oxygen breathing condition, as compared with the systemic arterial pO_2 , the retinal vasculature pO_2 measurements were significantly lower ($p \leq 0.002$), while the choroidal pO_2 was similar ($p = 0.7$). The pO_2 in the retinal vein was statistically lower than in all the other vasculatures ($p < 0.001$). Under 50% oxygen breathing condition, the chorioretinal pO_2 measurements were significantly lower ($p < 0.001$) than the systemic arterial pO_2 . The retinal venous pO_2 was less than the pO_2 measured in the retinal artery ($p < 0.001$), capillaries ($p = 0.06$), and in the choroid ($p = 0.003$).

Retinal arterial and venous pO_2 increased with increased fractions of inspired oxygen but at different proportional increments. Due to the difference in the rate of increase in the retinal arterial and venous pO_2 , the arterial-venous (A-V) difference was found to increase with increasing oxygen intake. The intrasubject variabilities determined from standard deviation of repeated measurements in the same eye, in the retinal artery, vein, capillaries, and choroid were 2, 3, 3, and 3 mmHg, during room-air breathing; 9, 3, 5, and 4 mmHg, during 50% oxygen breathing; and 3, 3, 1, and 3 mmHg, during 10% oxygen breathing conditions, respectively.

The mean pO_2 measurements in the chorioretinal vasculatures obtained before (baseline) and during infusion of N^{ω} -NLA are shown in Fig. 4. During infusion of N^{ω} -NLA, pO_2 in the retinal vasculatures remained relatively unchanged, whereas pO_2 in the choroidal vasculature was considerably reduced, as compared with baseline measurements. Measurements of pO_2 in the retinal artery, vein, capillaries, and choroid during infusion of N^{ω} -NLA are plotted as a function of measurements obtained at baseline (Fig. 5). The data points representing pO_2 measurements in the retinal vasculatures were adjacent to the identity line, indicating comparable values before and during N^{ω} -NLA infusion. The data points corresponding to choroidal pO_2 measurements were predominately below the identity line, denoting decreased choroidal pO_2 with the infusion of N^{ω} -NLA. Only one data point was in close proximity to the identity line, which represented the choroidal pO_2 measurement at N^{ω} -NLA infusion rate of 1 mg/min. The percent change in choroidal pO_2 at each N^{ω} -NLA infusion rate was calculated based on data obtained in two rats (Fig. 6). The choroidal pO_2 decreased markedly with each incremental increase in N^{ω} -NLA infusion rate, in the range 1–6 mg/min. There was little further change in the choroidal pO_2 at N^{ω} -NLA infusion rates above 6 mg/min.

DISCUSSION

Optimal oxygenation of the retinal tissue is ensured by the presence of a dual vascular supply, widespread intracranial anastomoses,³⁰ and the ability of the chorioretinal vascular circuit to regulate blood flow by means of both intrinsic autoregulatory and extrinsic autonomic feedback loops.³¹ Because vascular pO_2 is dependent on both the supply of oxygenated blood and the extraction of oxygen by the retinal tissue, technologies that provide direct measurement of

chorioretinal pO_2 can be invaluable to supplement knowledge of normal retinal vascular function and retinal pathologies associated with hypoxia or lowered blood flow. In the current study, an optical imaging system was developed for the measurement of pO_2 separately in the retinal and choroidal vasculatures. The feasibility of the system was established by demonstrating an increase in the retinal and choroidal pO_2 with increased fractions of inspired oxygen and a decrease in the choroidal pO_2 with systemic infusion of an NOS inhibitor.

With increasingly rich oxygen breathing conditions, pO_2 was observed to increase in the retinal arteries, veins, and capillaries, as well as in the choroid. Retinal arterial and venous pO_2 measurements increased with the fraction of inspired oxygen, in agreement with the results of a previous study that investigated pO_2 measurements in the mouse and rat.¹³ A 60% change in systemic arterial pO_2 corresponded with a 43% change in the choroidal pO_2 measurement, similar to a previous study that measured a 49% change in choroidal pO_2 with oxygen microelectrodes.³² In the current study, the A-V difference in the retinal circulation also increased with increasing fractions of inspired oxygen, which may be due to autoregulation. It is known that both retinal veins and arteries constrict in response to hyperoxic challenge.^{33–35} Consequently, assuming constant oxygen consumption, the lower resultant blood flow through the retinal vasculature will presumably result in a higher fractional extraction of oxygen and hence an increase in the A-V difference. At pO_2 over about 100 mmHg, hemoglobin is saturated with oxygen. At pO_2 under 100 mmHg, oxygen consumption removes oxygen from oxy-hemoglobin. Thus, a given amount of oxygen extraction produces a smaller drop in pO_2 in the blood when there is a release of oxygen from hemoglobin. During hyperoxic challenge, the dissolved pool of oxygen provides a larger proportion of the oxygen consumed by the retinal tissue than with lower levels of inhaled oxygen. Normal oxygen consumption will hence cause a greater change in oxygen partial pressure at higher arterial pO_2 . The difference between systemic and retinal arterial pO_2 was found to increase during hyperoxia in the current study. This finding may be attributed to previously reported vaso-constriction and lowered retinal arterial blood flow during hyperoxia,³⁶ which tend to increase the pO_2 drop along the arteries.³⁷

In the current study, the systemic arterial pO_2 , under room-air breathing conditions, was found to be 53 mmHg, which is lower than measurements obtained in previous studies.^{38,39} The apparent hypoxic condition of the rats in our study may be attributed to the respiratory depressant effect of the anesthetics, because the rats breathed spontaneously and were not intubated or ventilated. Despite the lower than normal arterial pO_2 , changes in systemic arterial pO_2 were induced, in accordance with varying fractions of inspired oxygen. When the systemic arterial pO_2 was above 100 mmHg, the choroidal pO_2 was found to be approximately one half of the systemic arterial pO_2 , in agreement with a previous study that used oxygen microelectrodes.³²

With infusion of N^G -NLA, a marked decrease in choroidal pO_2 was demonstrated, which may be attributed to inhibition of the synthesis of NO that maintains a vasodilatory influence on the choroidal vasculature.¹⁶ Previous studies have demonstrated a decrease in choroidal blood flow with intravenously administered systemic inhibitors of NOS.^{19,40–45} A decrease in choroidal blood flow might be expected to lead to a reduction in the choroidal pO_2 , as was observed. In the current study, the retinal vascular pO_2 remained relatively constant with NOS inhibition, consistent with previous studies that have reported no change in the retinal blood flow.^{40–42,46} However, other studies have shown a decrease in retinal blood flow with NOS inhibition.^{21–24} Although, NO functions as a vasodilator and has been shown to control the basal arteriolar tone in the inner retina,⁴⁷ the presence of a retinal tissue-derived vasorelaxant^{48,49} may counteract the effect of NOS inhibition, yielding a lack of alteration in the retinal vasculatures pO_2 .

Chorioretinal pO_2 is expected to vary due to changes in blood flow and also based on the rate of oxygen extraction by the retinal tissue. Hence, the advantage of direct measurement of pO_2 over blood flow measurements is that it provides a more complete means for evaluating the metabolism of retinal tissue. A dynamic equilibrium exists in normal neural tissue between perfusion and oxygen consumption. As with other neural tissue, in pathological states, retinal tissue will exhibit inadequate matching of oxygen consumption with perfusion. For example, in acute retinal embolic disease, the acute phase will be marked by a state of “misery perfusion,” or inadequate perfusion that does not match the consumption requirements of retinal tissue. In the subacute phase, after death of retinal tissue, the demand for oxygen will be reduced, resulting in a state of “luxury perfusion.” These states will manifest changes in the venous pO_2 that will depend on the metabolic state of the retinal tissue. Hence, direct measurements of pO_2 may provide information on the matching between oxygen consumption and delivery, which is essential for investigating whether altered blood flow in the chorioretinal vasculatures is the cause of or is a consequence of retinal diseases and pathologic conditions.

One potential limitation of our technique is the presence of autofluorescence from the retinal pigment epithelium that produces a contaminating intensity signal with no phase shift, resulting in smaller measured phase shifts and correspondingly larger pO_2 values. Because the phase shift due to autofluorescence is constant, it only affects the absolute values of pO_2 measurements. However, its contribution to the pO_2 measurement will likely be greater under high pO_2 conditions, due to lower phosphorescence signal. This factor is not expected to impact the measurements of moderate pO_2 changes in the physiological range in the normal or diseased retina. Recently, the phototoxic effect of the oxygen probe has been studied.⁵⁰ Although, the retinal irradiance used in the current study was below the level that produced tissue damage, repeated laser exposures have the potential to induce phototoxic injury. The application of this technique is currently limited to study oxygenation changes in animal models of retinal diseases, because the safety of the oxygen probe for use in human subjects has not yet been established.

In summary, an optical imaging system for quantitative measurement of chorioretinal pO_2 was developed, and its feasibility to determine pO_2 changes separately in the retinal and choroidal vasculatures was established. The application of this technique may allow better understanding of choroidal and retinal oxygen dynamics in physiologic and pathologic states.

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FIGURE 1.

Schematic diagram of the optical section phosphorescence imaging instrument developed for measurement of oxygen tension in the chorioretinal vasculatures.

FIGURE 2.

(A) Optical section phosphorescence image shows the retinal and choroidal vasculatures displaced in depth. The retina and choroid are on the left and right side of the image, respectively, as indicated by arrowheads. Retinal artery, vein, capillaries, and choroid are indicated by the arrows. (B) A set of six phase-delayed images are shown with a retinal vein demarcated as the region of interest. Phosphorescence intensity was measured within the region of a retinal vein in each of the images and plotted as a function of phase delay. The phase shift (delineated by the vertical line) was determined from the best-fit sinusoidal curve, corresponding to an oxygen tension measurement of 29.5 mmHg.

FIGURE 3.

Oxygen tension measurements in the systemic artery, retinal artery, vein, capillaries, and choroid obtained in six rats under varying fractions of inspired oxygen. The error bars represent the standard error of the measurement means.

FIGURE 4.

Oxygen tension measurements in the retinal artery, vein, capillaries, and choroid obtained in five rats before (baseline) and during N^{ω} -NLA infusion. The error bars represent standard error of the means of measurements.

FIGURE 5.

Chorioretinal oxygen tension (pO_2) measurements obtained during N^{ω} -NLA infusion plotted as a function of measurements obtained prior to N^{ω} -NLA infusion (baseline). The data points corresponding to pO_2 measurements in the retinal vasculatures were adjacent to the identity line, indicating comparable values before and during N^{ω} -NLA infusion. The data points corresponding to the choroidal pO_2 measurements were predominately below the identity line, denoting decreased choroidal pO_2 during infusion of N^{ω} -NLA.

FIGURE 6.

The percent change in the choroidal oxygen tension is plotted as a function of N^{ω} -NLA infusion rate based on data obtained in two rats.