Monitoring angiogenesis using a human compatible calibration for broadband near-infrared spectroscopy

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Abstract. Angiogenesis is a hallmark of many conditions, including cancer, stroke, vascular disease, diabetes, and high-altitude exposure. We have previously shown that one can study angiogenesis in animal models by using total hemoglobin (tHb) as a marker of cerebral blood volume (CBV), measured using broadband near-infrared spectroscopy (bNIRS). However, the method was not suitable for patients as global anoxia was used for the calibration. Here we determine if angiogenesis could be detected using a calibration method that could be applied to patients. CBV, as a marker of angiogenesis, is quantified in a rat cortex before and after hypoxia acclimation. Rats are acclimated at 370-mmHg pressure for three weeks, while rats in the control group are housed under the same conditions, but under normal pressure. CBV increased in each animal in the acclimation group. The mean CBV (%volume/volume) is 3.49% ± 0.43% (mean ± SD) before acclimation for the experimental group, and 4.76% ± 0.29% after acclimation. The CBV for the control group is 3.28% ± 0.75%, and 3.09% ± 0.48% for the two measurements. This demonstrates that angiogenesis can be monitored noninvasively over time using a bNIRS system with a calibration method that is compatible with human use and less stressful for studies using animals.

Keywords: near-infrared spectroscopy; near infrared; hemoglobin; acclimation; cerebral blood volume; angiogenesis; hypoxia; brain.

1 Introduction

Noninvasive measurements of cerebral blood volume (CBV) can be used as a marker of vascular density.1,2 By quantifying vascular density, one can study the process of angiogenesis, or growing new blood vessels, which is a significant area of interest in the study of diseases such as cancer, stroke, and diabetes.3–5

Methods used to measure vessel density using CBV include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and single-photon emission computed tomography (SPECT).6–10 Although each method has strengths, they usually require injection of contrast agents and are relatively expensive to operate. This could potentially limit the number of times the measurements can be conducted. Hence a repeatable, inexpensive method capable of quantifying changes in CBV is desirable, for both research as well as clinical practice.

We showed previously that quantitative near-infrared spectroscopy (qNIRS) via a broadband NIRS (bNIRS) system can be used to quantify tissue total hemoglobin ([tHb]) and CBV as a method of monitoring angiogenesis in rat brain.11 bNIRS measures a broad near-infrared (NIR) spectrum and uses modeling of the attenuation spectra to measure tissue deoxyhemoglobin concentrations.1,11 A common method of calibration for quantifying [tHb] and saturation has been called the anoxia method, which requires subjects to breathe 0% oxygen (anoxia) for approximately 50 to 60 s.1,11

This method assumes that a brief pulse of anoxia will convert all of the hemoglobin into the deoxygenated form, allowing for [tHb] to be calculated by quantifying tissue deoxyhemoglobin (HHb). CBV can then be calculated from [tHb] and large vessel hemoglobin concentration.

This type of calibration is not suitable for use in a wide range of conditions. For instance, anoxia may result in some level of hypoxic preconditioning12,13 and may influence the outcome of animal studies of stroke. It is also not suitable for studies on patients since breathing 50 s of anoxic gas would not be tolerated.

We showed previously that one could substitute the anoxia pulse method with a graded hypoxia method for calibration of bNIRS.14 Using the graded hypoxia calibration, [tHb] can be obtained by measuring the effects of a small, step-wise change in arterial oxygenation (S O2) on HHb concentrations. Since mild hypoxia is well tolerated, this method would broaden the applications of bNIRS. Although a previous study has validated its accuracy in measuring CBV under normal conditions,14 it has yet to be proven that this method is suitable for detecting angiogenesis.

Previous studies have demonstrated that chronic hypoxia exposure produces significant angiogenesis and increases in blood vessel density.12,15–18 We used chronic hypoxia exposure in rats as a means of stimulating angiogenesis. We show that quantifying angiogenesis in brain in a repeated, noninvasive fashion is possible with bNIRS using a graded hypoxia calibration method.
2 Methods

2.1 Broadband Near-Infrared System

The bNIRS system is custom-built using commercially available components, including an imaging spectrograph (Shamrock 303i, Andor Technology Inc., Northern Ireland), a broadband light source (Model 77501, Oriel Instruments Inc.), two multiple core silica optic fibers (Techen, USA), a charge-coupled device camera (DU420-BR-DD, Andor Technology Inc., Northern Ireland), and a data processor (Optix280, Dell, USA). After calibration using a reference neon spectrum, the NIR attenuation spectra were measured. The 740- to 810-nm region of the spectrum was assigned to HHb, and the 810- to 840-nm region was assigned to water. Absolute [HHb] was determined via the second differential method.11 It was assumed that the rat brain consisted of 80% water, and that chronic hypoxia does not produce a change in water content of the rat’s brain.18

Custom software that integrated the hardware components was used to perform data collection and analysis. The second differential analysis of the attenuation spectra was used to quantify HHb per volume of water.11,19

2.2 Graded Hypoxia Calibration

The graded hypoxia method is a modified version of previously described methods.10,20–23 This calibration method assumes that if changes in arterial hemoglobin saturation (SpO2) are brief, small, and gradual, there will be no accompanying changes in cerebral metabolic rate for oxygen (CMRO2), cerebral blood flow (CBF), and CBV.24 Numerous studies have validated this assumption, showing that CBV, CMRO2, and CBF stayed constant during moderate hypoxemia and changed only under conditions of extreme arterial O2 deficiency when SpO2 dropped below 0.75.20,25,26 The derivation of the calibration method is shown below and is detailed in a previous study.14 NIRS is sensitive to largely to microvessel hemoglobin. Tissue hemoglobin oxygen saturation (SpO2) measured by NIRS is composed of SpO2, capillary hemoglobin saturation, and venous saturation (StO2). It has been generally accepted that the contribution to blood volume is 20% arterial, 10% capillary, and 70% venous. Assuming the capillary contains equal amounts of arterial and venous blood, SpO2 can be determined by Eq. (1):27

\[ SpO_2 = 0.25 \times S_aO_2 + 0.75 \times S_vO_2, \]  

where SpO2 is the tissue hemoglobin oxygen saturation, S_aO2 is the arterial oxygen saturation, and S_vO2 is the venous oxygen saturation.

The CMRO2 can be calculated using the Fick principle:28

\[ \text{CMRO}_2 = (S_vO_2 - S_aO_2) \times \frac{K \times \text{CBF} \times [\text{Hb}]}{\text{CBV}}. \]  

where [Hb] is the total hemoglobin concentration in a large blood vessel (g/L), CBF is the cerebral blood flow, and K is the carrying capacity of hemoglobin.

We can express the Fick Equation via SpO2 using Eqs. (1) and (2). SpO2 is the oxygen saturation of hemoglobin, defined by [HbO]/[Hb], where [HbO] is the tissue oxyhemoglobin concentration, and [Hb] is the sum of [HbO] and [HHb]. Since SpO2 = [HbO]/[Hb] and [Hb] = [HbO] + [HHb], SpO2 can be expressed as:

\[ SpO_2 = 1 - \frac{[HHb]}{[Hb]}, \]  

where [Hb] and [HHb] are the concentrations of tissue total hemoglobin and tissue deoxyhemoglobin measured by the bNIRS system, respectively.

Combining the three equations above, [HHb] can be expressed as follows:

\[ [HHb] = [tHb] \times (1 - SpO_2) \]  

\[ + \left(\frac{0.75 \times \text{CMRO}_2}{K \times \text{CBF} \times [\text{Hb}]} \times [\text{Hb}]\right). \]  

Equation (4) shows that [HHb] has a linear relationship with (1-SpO2); since SpO2 can be measured by a pulse oximeter, [Hb] can be determined from the slope of Eq. (4). After determining [Hb], SpO2 can also be determined from Eq. (3). This derivation is more completely described in Zhang et al.14

Since CMRO2, CBF, and [Hb] was assumed to not change during the procedure, the “0.75CMRO2/K × CBF × [Hb]” will be constant. Tissue total hemoglobin measured using this method will not be affected by the original values for CMRO2 and CBF since they do not affect the slope of this equation. An additional advantage of using this simplification is that the assumption concerning the relative compartment size of the arterial and venous system (25% arterial, 75% venous) does not influence the final value of [HHb] as the values are contained in the constant.

[tHb] can then be used to calculate cortical CBV (volume to volume) in the following way:

\[ \text{CBV}(\% v/v) = \frac{[\text{Hb}] \times k_1 \times [\text{Hb}]}{[\text{Hb}] \times R} \times 10^{-4}, \]  

where [Hb] is the large vessel total hemoglobin concentration (g/L) obtained from the blood sample, k1 is the molar mass of hemoglobin (64500 g/mol), and R is the ratio of small to large vessel hematocrit set at 0.61.29

3 Experimental Procedure

For three weeks, male Wistar rats (n = 7) were exposed to hypobaric hypoxia maintained at 370 ± 2.1 mmHg (mean ± S.D.) using a custom built hypoxic chamber. The control rats (n = 4) were housed under the same conditions as the hypoxia acclimated animals but not exposed to hypobaric hypoxia. CBV of all rats was determined before and after hypoxia acclimation using bNIRS. In order to minimize the influence of posthypoxic hypcapnia, which can stimulate cerebral blood flow,28 postacclimated animals were measured 24 h after removal from the hypoxic chamber. It has been shown that elevated CBF will also elevate CBV.30 Although we’d still be measuring an accurate CBV, a high steady state CBF would result in an overestimate of the change in CBV relative to the nonacclimated animals.

A mixture of 2% isoflurane, N2 (~68%), and O2 (~30%) was used to anaesthetize and ventilate the animals through a nose cone. A heated water bed and rectal thermometer was used to maintain body temperature at 37°C to 38°C. A pulse oximeter was used to measure heart rate and SpO2 (Model 8600; Nonin, Inc., USA).
A blood gas analyzer (Stat Profile CCX, Nova Biomedical Corporation, USA) was used to analyze blood (200μL) obtained from the tail vein. Depilatory cream (Nair Church & Dwight Co., Inc. USA) was used to remove fur on the head. Along the midline and approximately 5-mm anterior of the interaural line, the bNIRS optode was pressed onto the scalp, with 7-mm lateral distance between the source and detector. The cortical area, retrosplenial dysgranular cortex and lateral parietal association cortex were the primary components of the detected area.

Following the baseline measurement, the inspired oxygen fraction (FiO₂) was decreased by decreasing O₂/N₂ gas ratio so that hypoxia is induced. SₐO₂ was initially reduced by 5% and sustained briefly; within 5 to 10 min, SₐO₂ and FiO₂ were slowly restored to baseline. The reduction and step-wise increase of the FiO₂ was controlled by a computer (SAR 830/P Ventilator, IITC Life Science Inc., USA). The pulse oximeter was time-synced with the bNIRS system by a data acquisition and analysis workstation (MP150 system, BIOPAC Inc. USA).

All experiments has been approved by the Animal Care Committee at the University of Calgary and conformed to guidelines established by the Canadian Council on Animal Care. A paired t-test was used to compare CBV before and after acclimation for both the experimental and the control groups, where p < 0.05 was considered significant.

4 Results

Broadband attenuation spectra were obtained from the head of the anesthetized rat at the same time as data were obtained on arterial hemoglobin saturation using a pulse oximeter. The arterial hemoglobin saturation was reduced to 5% to 6% below baseline values, and then restored gradually. The graded hypoxia calibration took approximately 11 min. Figure 1 shows a representative time course of [HHb] and the calculated 1 − SₐO₂ as the inspired oxygen fraction (FiO₂) was increased to normoxia in a step-wise fashion. In Fig. 1, the initial SₐO₂ declined by 6%. Based on Eq. (4) a plot of [HHb] versus (1 − SₐO₂) will result in a slope that equals [Hb]. A sample dataset is shown in Fig 2. These data were from the same animals used in Fig. 1.

The calculation of CBV [Eq. (5)] requires [Hb], [HHb], and an estimate of large to small vessel hematocrit. Table 1 shows the physiological variables used in addition to the NIRS measure of [HHb], for the calculation of CBV; these parameters were measured by analyzing the blood from a tail vein using a blood gas analyzer. There were significant differences between the control and acclimation groups in large vessel hemoglobin content and hematocrit.

Table 1 shows the increase in CBV in the cortex for all animals due to a three-week chronic hypoxia exposure (n = 7). Changes in CBV for control and hypoxia-acclimated rats are illustrated in Fig. 3. The mean CBV in the hypoxia acclimated group was increased by 36% (p < 0.001). There were no significant differences in CBV values between the two time points in the control group (n = 4, p > 0.05). There was a 12% increase in SₐO₂ (p < 0.05) in the hypoxia acclimated group. There were no significant differences in SₐO₂ in the control group.

5 Discussion

The graded hypoxia calibration has a great advantage compared to the anoxia pulse technique used to quantify bNIRS signal. A small, 5% to 6% decline in SₐO₂ provides a reasonable dynamic range, which is well tolerated by humans. This method is less stressful compared with the anoxia method where the subjects have to breathe 0% oxygen for an extended period.

The graded hypoxia method of calibration was slightly more difficult to implement compared with the anoxia pulse method. The graded hypoxia method requires approximately 10 min more time than anoxia pulse method; it requires a sensitive pulse oximeter and a way of controlling inspired gas. For repeated studies using many subjects, one could even use premixed gases, replacing the need for a gas mixer. In addition, the [Hb] needs to be known. For the general population, this value could be assumed to be 15 g/dL. In the current study, hypoxia is known to stimulate production of erythrocytes and increase hematocrit and [Hb]. In such studies, where [Hb] may change, a large vessel (venous or arterial) blood sample is needed to quantify [Hb] to control for any variation in hematocrit or [Hb].

The model is based on Eq. (4), which has grouped five variables into one constant that is assumed not to change during the calibration. In order to gain some confidence that the model is valid, we substituted for the variables using data on rat brain for...
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CBF, CMRO₂ (Ref. 31) as well as [Hb], hemoglobin carrying capacity (1.34 mL O₂∕gH b), and [Hb]. With this substitution, the calculated intercept is 13.6 versus the observed average value of 12.2 ± 3.96 (mean ± SD). The calculated value falls within one standard deviation of the values in observed in our study. The slight deviation from the expected value is likely to be accounted for by the fact that literature values were used. The model can be derived as on physiological principles, but this calculation provides additional evidence of its validity. For the purposes of actually calculating the CBV, these parameters do not need to be measured because they do not influence the slope.

A preacclimation CBV value of 3.41% ± 0.54% was determined, which is in good agreement with the 3.8% measured using radioactively labeled red blood cells and plasma, 3.26% measured with the anoxia calibration method using a bNIRS system, and 3% measured with MRI using contrast agents.

We were able to detect increases in vascular density in all animals exposed to chronic hypoxia but saw no significant changes in the control group (Fig. 1). The 36% percent increase is in good agreement with a 31% increase in a previous study with bNIRS, which used anoxia to quantify hypoxia induced changes in CBV, a 29% and 30% increase in capillary density, both measured using histology. This is lower than the 57% increase previously reported, which may be explained by the altitude difference at which the experiments have been carried out. The good agreement with previous literature suggests that the graded hypoxia method is sensitive enough to monitor changes in CBV.

After hypoxia acclimation, the hemoglobin saturation in the microvasculature (S₅O₂) increased (Table 1). The effect of acclimation on tissue oxygenation has been modeled and predicts an increase in hemoglobin saturation. Direct measurements of tissue PO₂ undertaken at sea level reported a 238% increase in tissue PO₂ (Ref. 16). A study using the same technique carried out in the Calgary reported a PO₂ increase of 35%. Using the rat oxy-hemoglobin dissociation curve, the predicted change in PO₂ with a change in hemoglobin saturation from 72% to 82% would be 51 to 64 mmHg (25%). This is lower than the observed changes in PO₂, but that is expected given the fact that increased capillary density will also contribute to the increased tissue PO₂ (Ref. 34).

This calibration method can be applied to many types of diseased animal models, including stroke. This calibration can also be used with a wide variety of drugs, as long as CMRO₂ and CBF are maintained at a steady state during the calibration procedure. The absolute values of CMRO₂ and CBF before and after the calibration method will not affect the calculation of tissue total hemoglobin, as they are contained in the constant and do not have an impact on the slope. However, under conditions where the CMRO₂ or CBF is subject to change in the middle of the calibration, such as during an episode of seizure, this method will not work since the slope of the regression will be affected.

There is a limitation with estimating CBV using any noninvasive NIRS method. The fact that NIRS signals originate from both brain and the skull, which can induce error, as the CBV obtained is the average blood volume in the brain and skull. In conditions where angiogenesis will only occur in the brain but not in the skull, this method can underestimate the calculated CBV. However, this will only reduce the sensitivity of this method in detecting CBV changes.

There are other quantitative NIRS methods, such as the frequency domain (FD) and the time resolved (TRS) NIRS, which can provide quantitative hemoglobin data. The FD method operates on the theory that changes in source detector distance will result in changes in the time average (Rₐ), modulation amplitude (R₈), and phase shift (R₉) of a sinusoidal wave. By varying the source detector distances and plotting them against each of these three parameters, µₐ and µₛ can be solved using the slopes of these three regressions. The TRS method utilizes the relationship between the amount of

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**Table 1** Physiological parameters and calculated CBV (mean ± SD).

| Condition            | Weight (g) | Hematocrit (%) | [Hb] (g/l) | S₅O₂ (%) | CBV (%) |
|----------------------|------------|----------------|------------|----------|---------|
| Control n = 4        | Pre        | 356 ± 37       | 49.5 ± 5.7 | 165.0 ± 19.7 | 72.4 ± 5.1 | 3.28 ± 0.75 |
|                      | Post       | 447 ± 29       | 48.0 ± 3.9 | 164.8 ± 17.9 | 70.8 ± 1.0 | 3.09 ± 0.48 |
| Hypoxia Acclimation n = 7 | Pre | 327 ± 23       | 46.9 ± 4.6 | 156.5 ± 15.2 | 73.5 ± 3.8 | 3.49 ± 0.43 |
|                      | Post       | 381 ± 9        | 61.0 ± 4.4 | 202.4 ± 14.4 | 82.4 ± 5.3 | 4.76 ± 0.29 |

*Significantly different from the preacclimation values, p < 0.05.
Significantly different from the preacclimation values, p < 0.001.

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**Fig. 2** A representative linear regression between [Hb] and 1-S₅O₂ during the graded hypoxia process in one animal. The regression of the line is y = 91.38x + 11.4. The clustered data for 5O₂ is a result of the step-wise decline in inspired O₂. The slope of the line represents the total hemoglobin concentration (μM).
time it takes for photons to reach the detector (time of flight) and depth of the tissue penetrated. By analyzing the distribution of the time of flight for the diffusely reflected light, one is able to determine whether the tissue is more or less absorbing or fluorescing than normal. This would include the possibility of using the system for simultaneous detection of multiple absorbing or fluorescing compounds. This would include the possibility of using the system for simultaneous detection of multiple absorbing or fluorescing compounds. The graded hypoxia calibration provides a third method of quantification for use in broadband systems.

Quantification of total hemoglobin as a marker of blood volume in human subjects using a NIRS system has significant advantages. Compared with MRI, CT, and PET, the low cost and noninvasive nature of NIRS allows it to be used in a wide range of applications. Furthermore, since NIRS is portable, measurements can be performed at bedside, which greatly benefits patients who cannot be mobilized. Broadband NIRS has some advantages compared with other qNIRS systems. Since a broadband system utilizes the entire absorption spectrum, it leads to the possibility of using the system for simultaneous detection of other absorbing or fluorescing compounds. This would include cytochrome oxidase, an important indicator of metabolism, fibrin formation, which play a key role in thrombosis, and even activated endothelia binding peptides labeled with NIRS fluorescent probes to monitor inflammation.

This study illustrates that changes in CBV as a result of angiogenesis can be accurately quantified over a time-course in individual subjects using a NIRS system in a manner that can be translated to humans and is less stressful for animal studies.

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