Reliability of Muscle Blood Flow and Oxygen Consumption Response from Exercise Using Near-infrared Spectroscopy

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Running head: Local skeletal muscle blood flow and oxygen consumption assessment

Keywords: microvascular, hemodynamics, oxygen consumption

Word Count: 6287

Reference Count: 37

Subject Area: Human/environmental and exercise physiology

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This is the pre-peer reviewed version of the following article: Lucero et al. (2017). Reliability of Muscle Blood Flow and Oxygen Consumption Response from Exercise Using Near-infrared Spectroscopy. *Experimental Physiology.* – which has been published in final form at https://doi.org/10.1113/EP086537. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

New Findings

- What is the central question of this study?

Continuous wave near-infrared spectroscopy coupled with venous and arterial occlusions offers an economical, non-invasive alternative to measuring skeletal muscle blood flow and oxygen consumption, however its reliability during exercise has not been established.

- What is the main finding and its importance?

Continuous wave near-infrared spectroscopy devices can reliably assess local skeletal muscle blood flow and oxygen consumption from the vastus lateralis in healthy, physically active adults. The patterns of response exhibited during exercise of varying intensity agree with other published results using similar methodologies, meriting potential applications in clinical diagnosis and therapeutic assessment.

Abstract

Near-infrared spectroscopy (NIRS), coupled with rapid venous (VO) and arterial occlusions (AO) can be used to non-invasively estimate resting local skeletal muscle blood flow (mBF) and oxygen consumption ($m\dot{V}O_2$), respectively. However, the day-to-day reliability of mBF and $m\dot{V}O_2$ responses to stressors such as incremental dynamic exercise has not been established. *Purpose:* To determine the reliability of NIRS derived mBF and $m\dot{V}O_2$ response from incremental dynamic exercise. *Methods:* Measurements of mBF and $m\dot{V}O_2$ were collected in the *vastus lateralis* of twelve healthy, physically active adults [7 m and 5 f; 25 y (SD 6)] over 3 non-consecutive visits within 10 days. After 10 mins rest, participants performed 3 mins of rhythmic isotonic knee extension (1 extension/4 s) at 5, 10, 15, 20, 25, and 30% of maximal voluntary contraction (MVC), prior to 4 VOs and then 2 AOs. *Results:* mBF and $m\dot{V}O_2$ proportionally increased with intensity (0.55 to 7.68 $ml \cdot min^{-1} \cdot 100ml^{-1}$ and 0.05 to 1.86 $mlO_2 \cdot min^{-1} \cdot 100g^{-1}$, respectively) up to 25% MVC where it began to plateau at 30% MVC. Moreover, a mBF/$m\dot{V}O_2$ $m\dot{V}O$ratio of ~5 was consistent for all exercise stages. The intra-class coefficient (ICC) for mBF indicated high to very high reliability for 10-30% MVC (0.82-0.9). There was very high reliability for $m\dot{V}O_2$ across all exercise stages (ICC 0.91-0.96). *Conclusion:* NIRS can reliably assess muscle blood flow and oxygen consumption responses to low-moderate exercise, meriting potential applications in clinical diagnosis and therapeutic assessment.

Introduction

Advancements in apparatuses and methods have permitted measurement and enhanced understanding of *in vivo* local skeletal muscle blood flow (mBF) (Rådegran, 1999; Casey *et al.*, 2008). Using techniques such as magnetic resonance imaging, contrast enhanced ultrasound, and intravascular tracer injection, the kinetics of flow through the microvasculature have been found to act differently of bulk flow through large conduit vessels (Vincent *et al.*, 2002), hence bulk flow may not accurately represent mBF (Harper *et al.*, 2006), the site of gas exchange, nutrient, and hormone delivery. In addition, mBF can vary throughout a single muscle (Quaresima *et al.*, 2004) and is tightly matched to metabolic demand (Joyner & Casey, 2015). Skeletal muscle oxygen consumption ($m\dot{V}O_2$) may be an important factor driving the regulation of mBF with previous evidence showing that $m\dot{V}O_2$/mBF ratio is maintained at a ratio of ~0.1 at rest and during exercise in healthy individuals (Vogiatzis *et al.*, 2015).

Characterizing mBF and $m\dot{V}O_2$ at rest and during dynamic exercise has become an important component of skeletal muscle hemodynamic and metabolic assessment and is necessary to fully comprehend blood flow regulation and dysregulation in humans. However, assessment of mBF during exercise is limited due to apparatus design, cost, technical skill, invasiveness, and functionality with various populations (Andersen *et al.*, 1985; Paunescu *et al.*, 1999; Rådegran, 1999; Casey *et al.*, 2008; Rudroff *et al.*, 2014). There exists a need for a reliable, non-invasive, and affordable technique that can effectively investigate mBF and $m\dot{V}O_2$ kinetics in various populations and disease states under real-world exercise conditions.

Continuous wave near-infrared spectroscopy (NIRS) is an emerging, affordable, and portable technology which enables the assessment of skeletal muscle hemodynamics through relative concentrations of oxygenated and deoxygenated hemoglobin. Currently, NIRS cannot differentiate between hemoglobin and myoglobin, but its contribution to the NIR signal is suggested to be less than 20% at rest, with the main contributor being hemoglobin (Ferrari *et al.*, 2011). Given that NIRS only measures changes in vessels smaller than 1-2 mm in diameter, it is ideal for assessing local skeletal muscle microcirculation (Mancini *et al.*, 1994). Combining NIRS with rapid venous (VO) and arterial (AO) occlusions to estimate mBF and $m\dot{V}O_2$, respectively, has been validated in the forearm (Van Beekvelt *et al.*, 2001; Cross & Sabapathy, 2015), calf (Casavola *et al.*, 2000), and VL (Quaresima *et al.*, 2004). However, this technique has been limited to rest and maximal isometric exercise states and no protocol has been developed to reliably assess both mBF and $m\dot{V}O_2$ response to specific steady state exercise. The purpose of this study was to determine the reliability of continuous wave NIRS derived estimates of mBF and $m\dot{V}O_2$ in the *vastus lateralis* (VL) in response to incremental dynamic knee extension exercise.

Methods

Ethical Approval

Twelve healthy, (7 males and 5 females) physically active (>3 h of moderate intensity exercise per week) adults participated in this study (Table 1). Participants were excluded if they were smokers, reported any known cardio-metabolic disorders, or were taking medications known to affect cardiovascular function. This study was not designed to examine potential sex differences; thus, menstrual cycle status was not controlled for in female participants. Ethical approval was obtained from the institutional Human Ethics Committee (HEC: Southern A Application) and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standard, except for registration in a database. All participants were informed of any risks and discomfort associated with the experiments prior to providing written informed consent.

Experimental Procedures

Each participant was tested on four different days in a dimly-lit, temperature controlled room [(20.5 ˚C (SD 0.8)]. On visit 1, participants were familiarized with the testing protocol and their maximum voluntary contraction (MVC) for a 90˚ isometric knee extension was obtained and reported as the maximum of three trials using an isokinetic dynamometer (Biodex Medical Systems, Inc. Shirly, NY, USA). To determine the MVC, the participant was seated on the dynamometer reclined to 70° giving a 110° hip angle, and the settings were adjusted so that the axial portion of the knee aligned with the axis of rotation on the dynamometer. When assessing mBF and m$\dot{V}O_2$, the participant's dominant leg was suspended at a 'neutral' position during occlusion. The 'neutral' position consisted of a knee-joint angle of 150^0 , which permitted a relaxed muscle length, thereby facilitating blood flow (Miura *et al.*, 2004). The non-working leg was suspended in neutral position throughout.

The experimental protocol was conducted on visits 2-4. All experimental tests occurred between the hours of 7-10 am following an overnight fast, having consumed only water, refraining from caffeine and supplement intake that morning. Participants also avoided strenuous physical activity and alcohol for 24 hours prior to experimentation. Using hemoglobin as an endogenous intravascular tracer, venous and arterial occlusions were used to estimate mBF and m$\dot{V}O_2$. Participants were seated on the dynamometer and while the NIRS probe was adhered to the skin and cuff was placed and tested for positioning and comfort. Following an additional 10 min of quiet seated rest, baseline measurements of mBF and m$\dot{V}O_2$ were assessed as the average of 4 VO and 2 AO measurements, respectively. Each occlusion was separated by 45 s of rest with VO durations of 15 s and AO durations of 15 and 30 s (Southern *et al.*, 2013). The participant then completed 6 stages of progressive intensity (5, 10, 15, 20, 25, and 30% of MVC) 90˚ rhythmic isotonic knee extension exercise (1 extension/4 sec) on the dynamometer (Watanabe & Akima, 2011).

For each intensity, the participant exercised continuously for 3 min prior to occlusions. This time was chosen as a balance between the likelihood of achieving steady state physiology without causing fatigue. Steady state was determined through pilot trials by a stabilizing of whole body oxygen consumption. Participants were instructed to contract to full extension and then allow their leg to fall back to the starting position. Immediately after the contraction phase of the last knee extension for each measurement point, as the leg was falling, the dynamometer was locked to hold the leg in the neutral position simultaneously as the cuff was inflated for 10 s. This caused a brief pause in exercise during which the occlusion occurred. Exercise was then resumed for 45 s to maintain steady state before another measurement was collected. As with baseline measurements, mBF and m$\dot{V}O_2$ were assessed as the average of 4 VO and 2 AO measurements, respectively (see Fig. 1A). Complete occlusion during AO at rest and exercise was verified with Doppler ultrasound in the femoral artery during pilot trials and confirmed during testing by the cessation of the pulsatile motion in the tHb signal. An index of perfusion change and tissue saturation index were also assessed during each stage.

Near-infrared Spectroscopy

A continuous wave NIRS device (PortaLite, Artinis Medical Systems BV, the Netherlands) emitted wavelengths of 760 and 850 nm to detect relative changes in concentrations of oxygenated hemoglobin [HbO_2] and deoxygenated hemoglobin [HHb], respectively, as well as total blood volume ([tHb] = [O_2Hb] + [HHb]). Absolute hemoglobin concentrations can be estimated, however, in this study only relative changes are used in calculations. Wavelengths were emitted from LEDs with an inter-optode distances of 3.5 cm, allowing for theoretical penetration distances of 1.75 cm (Chance *et al.*, 1992). A differential path-length factor of 4.0 was used to correct for photon scattering within the tissue, and data were collected at 10 Hz (Oxysoft, Artinis Medical Systems BV, the Netherlands). The NIRS probe was securely adhered to the skin parallel to the muscle fibers, about two-thirds from the top of the *vastus lateralis* over the muscle belly. A custom-made cover shielded the probe from ambient light while allowing it to move with the skin during contractions minimizing changes in contact pressure (Hamaoka *et al.*, 2011). The thickness of the muscle at this location, along with adipose tissue thickness, was determined using B-mode ultrasound (Terason, United Medical Instruments Inc., San Jose, CA, USA).

Local Skeletal Muscle Blood Flow

Estimates of mBF were assessed as the [ΔtHb] signal during VO, analyzed using simple linear regression as previously described (Van Beekvelt *et al.*, 2001; Cross & Sabapathy, 2015). Briefly, a tourniquet (Hokanson SC 10D, D. E. Hokanson, Inc., Bellevue, WA, USA) was placed as high as possible around the proximal thigh, minimizing patient discomfort and avoiding artefact motion in the NIRS signal. The tourniquet was rapidly (~0.5 s) inflated to a subdiastolic pressure (60-80 mmHg) occluding venous outflow without impeding arterial inflow, thus, causing venous volume to increase at a rate proportional to arterial inflow (Van Beekvelt *et al.*, 2001). After cuff inflation, there is a rapid, progressive fall in the rate of [ΔtHb] (especially during exercise), likely due to an increase in venous backpressure, diminishing the arteriovenous pressure gradient and stimulating the venoarterial reflex causing vasoconstriction of precapillary vessels (Rathbun *et al.*, 2008). As a consequence, inclusion of more than one cardiac beat has been shown to underestimate mBF (Cross & Sabapathy, 2015) (see Fig. 1B, C). Therefore, estimates of mBF were over the first cardiac cycle, defined using the pulsatile motion of the [tHb] signal. The slope of the [tHb] signal for each VO was averaged and converted into units of mL per min per 100 mL of blood (mBF ($mL \cdot min^{-1} \cdot 100\ mL^{-1}$) = $1/C \cdot [\Delta tHb]/\Delta t$) where $[\Delta tHb]/\Delta t$ is the average rate of tHb increase under VO (µM of $Hb \cdot s^{-1}$) and C is hemoglobin concentration in the blood, for which we assumed a value of 7.5 and 8.5 $mmol \cdot L^{-1}$for female and male participants, respectively (Van Beekvelt *et al.*, 2001). The molecular mass of hemoglobin (64.458 $g \cdot mol^{-1}$) and the ratio between hemoglobin and O_2 molecules (1:4) were accounted for.

Skeletal Muscle Oxygen Consumption

Estimates of $m\dot{V}O_2$ were calculated as the rate of change in the Hb difference signal ($[\Delta HbDif] = [\Delta HbO_2] - [\Delta HHb]$) during arterial occlusion (see Fig. 1D,E), analyzed using simple linear regression as previously described (Ryan *et al.*, 2012). Briefly, the tourniquet was rapidly (~0.5 s) inflated to a supra-systolic pressure (250-300 mmHg) to occlude both venous outflow and arterial inflow, completely arresting blood flow, resulting in an increase of [HHb] and simultaneous decrease in [HbO2] as oxygen is released from hemoglobin and consumed by the surrounding muscle tissue (Van Beekvelt *et al.*, 2001). After correcting for blood-volume changes (Ryan *et al.*, 2012), the slope of the [HbDif] signal for both AOs was averaged and converted into milliliters of O_2 per min per 100 grams of tissue ($m\dot{V}O_2$ ($mlO_2 \cdot min^{-1} \cdot 100g^{-1}$) = $abs[([\Delta HbDif/2] \cdot 60)/ (10 \cdot 1.04) \cdot 4] \cdot 22.4/1000$), assuming 22.4 L for the volume of gas (STPD) and 1.04 $kg \cdot L^{-1}$ for muscle density (Van Beekvelt *et al.*, 2001).

Local Skeletal Muscle Perfusion Change and Tissue Saturation Index

An estimate of relative local skeletal muscle perfusion was calculated as the relative average blood volume ([tHb] signal) for a given period. The [tHb] signal has been said to reflect microvascular blood-volume (Ijichi *et al.*, 2005) which reflects local O_2 diffusing capacity (Groebe & Thews, 1990). Since the [tHb] signal measures absolute changes from a set baseline, the resting value was set to 0 and the workload values were calculated as µM increases from rest. The tissue saturation index (TSI%) was calculated with manufacturer software using a spatially-resolved spectroscopy approach. The TSI% signal was averaged over the same period used for perfusion analysis.

After resting measurements of mBF and m$\dot{V}O_2$ were taken, the participant's leg was lowered to 90˚ knee-joint angle in preparation for the exercise protocol. The participant then continued to rest to allow the [tHb] signal to stabilize for 30 s to assess resting perfusion and TSI%. Estimated relative perfusion and TSI% were assessed as the average [tHb] and TSI% signal during the 2 s rest period between knee extensions (when the leg was relaxed at 90˚) of the last 8 extensions before the first VO.

Electromyography, Whole Body Oxygen Consumption & Heart Rate

To verify the exercise model elicited the desired metabolic increases, in a separate testing session surface electromyography (EMG), whole body oxygen consumption ($\dot{V}O_2$), and heart rate (HR) were measured in a subset of individuals (N=7). The EMG electrode (Telemyo DTS, Noraxon Inc., Scottsdale, AZ, USA) was placed over the NIRS probe location. To normalize the EMG activity signal prior to beginning the exercise protocol, the participant performed 3 MVCs and the peak forces were averaged and set to 100% activation. Integrated raw EMG signals were analyzed according to standard methods for knee extension exercise (Alkner *et al.*, 2000). To measure $\dot{V}O_2$, a breath-by-breath automatic gas exchange system (Vmax Spectra 29c, Sensormedics Corporation, Yorba Linda, CA, USA) was used, and HR was monitored using a wireless chest strap telemetry system (Polar Electro T31, Kempele, Finland).

Resting and exercise stage protocols were conducted like visits 2-4. However, during exercise after the first three minutes of knee extensions, the leg was not rested as occlusions were not required for assessing parameters. Baseline measures were assessed as the average value for the last minute of the resting period. Exercise parameters were assessed during the fourth minute of exercise. Exercise EMG activity was assessed as the average rectified maximum activity for the last eight contractions for each stage. Exercise $\dot{V}O_2$ and HR data were expressed as the average value for the last 60 seconds (fourth minute) of each stage.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences version 21 (SPSS, Inc., Chicago, Illinois). All data are reported as means with 90% confidence intervals, unless otherwise specified. The NIRS parameters were analyzed to test the effects of intensity and visit order using a two-way repeated measures analysis of variance (ANOVA)... Mechanistic inference testing for substantial differences between intensities were calculated from a published spreadsheet using generated p-values (Hopkins, 2007), with likelihood thresholds of 50% (possible), 75% (likely), 95% (very likely), and 99% (most likely) chance of substantial change. Values for mBF as a function of $m\dot{V}O_2$ were assessed for linearity using linear regression to test goodness of fit as a coefficient of determination (R^2). Likelihoods for correlations using magnitude based inference was used to test individual parameters against exercise intensity and to each other using 95% confidence limits with 0.2 as the threshold for smallest magnitude threshold for differences or change scores (Hopkins, 2007).

Reliability statistics were calculated with the log transformed raw data using published spreadsheets (Hopkins, 2015) as described previously (Hopkins *et al.*, 2009). The typical error (i.e. standard error of measurement) defined as $SD/\sqrt{2}$ where *SD* is the standard deviation of the change score for all participants. Test-retest reliability statistics calculated include the intra-class correlation coefficient (ICC), standardized typical error (STE), percentage coefficient of variation (%CV) and percentage of the smallest effect (%SE). The ICC gives visit to visit reproducibility for a given intensity and was calculated as $1\text{-}sd^2/SD_b^2$ where the *sd* is the typical error and SD_b the mean between-participant standard deviation. Thresholds of 0.20 (low), 0.50 (moderate), 0.75 (high), 0.90 (very high), and 0.99 (nearly perfect) reliability for sample populations were used. The STE gives the random error in the calibrated value and is interpreted using thresholds of 0.1 (small), 0.3 (moderate), 0.6 (large), 1 (very large), and 2 (extremely large) (Hopkins *et al.*, 2009). The typical error as a percentage is shown as CV (%). The SE (%) represents the percentage above or below the measured value required for the smallest worthwhile effect given by $0.2 \cdot SD_p$ where the SD_p is the pure between-subject standard deviation calculated as above.

Results

Mean values and inferences for mBF and $m\dot{V}O_2$, relative perfusion, and TSI% are shown in Fig. 2. Mean values for mBF and $m\dot{V}O_2$ were most likely (i.e., 99% chance) substantially greater than resting across all intensities. For both mBF and $m\dot{V}O_2$, mean values for all intensities were substantially greater than the previous intensity except for 30% MVC. Mean mBF correlated linearly with exercise intensity, and was directly proportional to $m\dot{V}O_2$ (y = 3.75x + 0.5384; R2 = 0.8195 - 0.9814 (Fig. 3A). The mean $m\dot{V}O_2$/mBF ratio was 0.045 at rest and varied from 0.104-0.132 for all exercise stages (Fig. 3B). Mean values for perfusion change from rest at 15-30% MVC were substantially greater to resting, however, only trivial increases to the previous intensity were seen in 10-30% MVC. Mean values for TSI% at 5, 10, and 30% MVC were substantially less than resting. Mean values for EMG, $\dot{V}O_2$, and HR during exercise increased substantially from resting (Fig. 4). For all three parameters, no substantial increase was observed at 10 and 25% MVC from the previous exercise intensity. There was a likely substantial increase in HR at 30% MVC, but not in EMG and $\dot{V}O_2$. All seven parameters were most likely substantially correlated with % MVC and to each other (99.7%-100% likelihood). No visit order effect was observed for all NIRS parameters.

Reproducibility for all NIRS parameters is shown in Table 2 as statistic value, with upper and lower 90% confidence limits available as supporting information. For mBF, the ICC indicated moderate reliability (0.69) at 5% MVC, but high reliability at rest and across all other intensities (0.82-0.89) with very high reliability at 25% MVC (0.9). The STE was moderate for rest and all exercise stages (0.35-0.59). It was lowest (best) for 20 and 25% MVC (0.37 and 0.35, respectively) and highest (worst) for 5 % MVC (0.59). The CV varied from 20.2 – 31% and was lowest (best) for 10-25% MVC (20.9-24.8%). The SE varied from 5.5-10.2%. For $m\dot{V}O_2$ the ICC indicated moderate reliability at rest (0.58) and very high reliability across all exercise stages (0.91-0.96). The STE was moderate at rest (0.58) and 5-20% MVC (0.31-0.34), and low (best) for both 25 and 30% MVC (0.22). The CV was 50.4% at rest, 20-22.6% for 5-20% MVC, and 13.5-14.0% for 25-30% MVC. The SE varied from 9.2-13.3% for all exercise stages.

Discussion

The purpose of this study was to determine the reliability of continuous wave NIRS derived estimates of mBF and $m\dot{V}O_2$ in the *vastus lateralis* during short intermittent pauses from dynamic exercise. Using occlusion methodology (i.e. AO and VO) combined with isotonic knee extensions at specific intensities, according to the ICC values, the current study found high to very high reliability from 10-30% MVC, and moderate reliability at 5% MVC. Comparing absolute estimates of mBF and $m\dot{V}O_2$ with previously reported values is difficult due to differences in exercise modality, muscle groups measured, and units used to express values. However, patterns of response in mBF and m$\dot{V}O_2$ to exercise intensity are in agreement with established results (Joyner & Casey, 2015), and the m$\dot{V}O_2$/mBF ratio is also consistent with previous findings (Vogiatzis *et al.*, 2015). Compared to other techniques, NIRS offers reliable, non-invasive application to real-world exercise modalities, and can be used on a wide variety of clinical populations (Paunescu *et al.*, 1999; Rådegran, 1999).

The relationship between mBF and mVO2 was consistent for all exercise intensities and within range of published mBF/m$\dot{V}$O2 ratios of ~5 (Whipp & Ward, 1982; Richardson *et al.*, 1995; Kalliokoski *et al.*, 2005), showing a tight match of mBF to m$\dot{V}O_2$ during steady-state exercise. For both mBF and m$\dot{V}O_2$, no substantial increase was seen from 25-30% MVC, as well as 20-30% MVC for EMG activity and $\dot{V}O_2$. Taken together, the current results suggest that maximal recruitment and/or fatigue developed in the primary *vastus lateralis* muscle fibers rprompting recruitment of accessory and additional muscle fibers in the quadriceps to sustain contractions (Komi & Tesch, 1979; Vøllestad, 1997). In support, many participants [N=8] appeared to have greater *rectus femoris* use at the higher intensities. Therefore, to obtain more accurate steady state estimates of mBF and m$\dot{V}O_2$ at specific intensities, it is recommended that in future trials, only 2 workloads between 10-25% MVC be tested in succession with adequate rest in between, depending on the population being tested. For example, 15 and 25% MVC may be ideal for physically active to athletic populations, but future trials will be needed to characterize intensities that can be maintained in clinical populations while achieving reproducible results.

Comparing the absolute estimates of mBF to those from other studies is difficult because a) mBF is heterogeneous across the muscle and can vary widely depending on the region measured, b) exercise modality used, and c) the units used to report mBF. Using a similar VO technique with a frequency-domain NIRS device, Quaresima *et al.* (2004) estimated blood flow to increase from 0.3-0.5 mL·min^{-1}·100 mL^{-1} to 1.4-2.1 mL·min^{-1}·100 mL^{-1} across the *vastus lateralis* from rest to maximal isometric exercise. Although the exercise modality differs to the current study, the resting value is within range of the current study and the exercise value for maximal isometric contraction is lower. Comparing indocyanine green injection and ^{133}Xe, Boushel *et al.* (2000) measured regional mBF in the calf during incremental plantar-flexion exercise to 9 watts, and found similar mBF values between the techniques concluding that mBF rose from about 2.2 mL·min^{-1}·100 mL^{-1} to 15.1 mL·min^{-1}·100 mL^{-1}. Although these estimates are larger than those reported in the current study, the muscle group and exercise modality differ. However, like the current study, the authors found increases in mBF to be proportional to workload.

Using thermodilution and dynamic knee extension exercise at 60 rpm to peak power, Rådegran et. al. (Rådegran *et al.*, 1999) found peak knee extensor mBF and m$\dot{V}O_2$ to be 246.2 ± 24.2 mL·min^{-1}·100 g^{-1} and 34.9 ± 3.7 mL·min^{-1}·100 g^{-1}, respectively, which is substantially higher than the current study. However, the exercise modalities differed significantly, in that the current study only went to 30% MVC, allowed for greater rest between contractions, and the force was only exerted at 90˚ rather than throughout extension, which would isolate a lower mass of contracting muscle (Joyner & Casey, 2015). Moreover, the current study measured one region within one knee extensor, the *vastus lateralis*, which has been shown exhibit ~57% mBF heterogeneity and to have ~20% less blood flow than the *vastus intermedius* during knee extension exercise (Rudroff *et al.*, 2014). In the previous study, using positron emission tomography, the authors found mBF in the *vastus lateralis* to be 6.21 ± 1.96 and 9.77 ± 3.82 mL·min^{-1}·100 g^{-1} at 2 and 12 min, respectively, of sustained isometric contraction at 25% MVC in young men, which is within range of the current study. In addition, EMG activity was also within range of the current study.

Limitations and Future Direction

Future application of the current protocol should consider a) timing of cuff inflation, b) addition of ECG monitoring and individual blood sampling, and c) concurrent monitoring of additional non-invasive measurements for assessing the entire oxygen cascade. Firstly, since VO can only be inflated between contractions, the resulting tHb slope reflects post-contraction values and not exercise *per se* (Rådegran, 1999). However, immediate post-exercise mBF has been shown to increase in proportion to exercise intensity (Kagaya & Homma, 1997) and reflect the mBF response to exercise (Quaresima *et al.*, 2004). Therefore, the low-pressure occlusion must be rapid (~0.5 s) and inflate immediately upon cessation of exercise. Secondly, the current study was not able to collect ECG or individual hemoglobin concentrations, however it is encouraged as time aligning the VO to cardiac cycles may increase reproducibility and individually sampled hemoglobin concentrations will enhance the accuracy of absolute mBF rates. In addition, synchronizing cuff inflation with ECG trace to occur at the same point within the cardiac cycle may standardize the attenuation effects of the VO on mBF. Lastly, concurrent non-invasive monitoring of additional parameters to assess the entire hemodynamic cascade may prove useful for mechanistic and pathological determinants.

Ensuring high reproducibility of NIRS derived measurements within a single subject is a critical step in the use of NIRS for clinical diagnosis. Our results have characterized reliability for estimating relative changes immediate post exercise mBF and $m\dot{V}O_2$ across a wide range of prevailing arterial inflows and O_2 consumption rates that can be used to estimate sample size and incorporated into future experimental design. Using a standard protocol to compare measurements of mBF and $m\dot{V}O_2$ during exercise in trained, untrained, and diseased populations will enhance our understanding of muscle physiology, mBF regulation, and disease pathogenesis. More research is required to understand the effects of exercise and muscle contraction on: a) the NIR light pathlength, b) changes in blood hemoglobin during exercise and its affect on mBF measures, c) and the contribution of myoglobin to the NIR signal at various exercise stages for the determination of absolute values of mBF and $m\dot{V}O2$. Future research should compare reliability and signal responses of continuous wave NIRS devices to other NIRS technologies, as well as assess the reliability and validity of using occlusions during varying exercise modalities and intensities compared to other leading techniques (Rådegran, 1999; Krix *et al.*, 2005; Duerschmied *et al.*, 2006; Partovi *et al.*, 2012; Pollak *et al.*, 2012).

Conclusion

In summary, continuous wave NIRS devices can reliably assess mBF and $m\dot{V}O_2$ within the microvasculature of the *vastus lateralis* during intermittent pauses from dynamic exercise in healthy, physically active adults. The relative patterns of response for mBF, and $m\dot{V}O_2$ during incremental exercise and mBF/$m\dot{V}O_2$ ratio agree with other published results using similar methodologies. Using NIRS to assess and characterize local parameters of skeletal muscle hemodynamics and metabolism during rest and exercise opens new research paradigms for the investigation of mBF regulation in health and disease with potential applications in clinical diagnosis and therapeutic assessment.

Figures and Tables

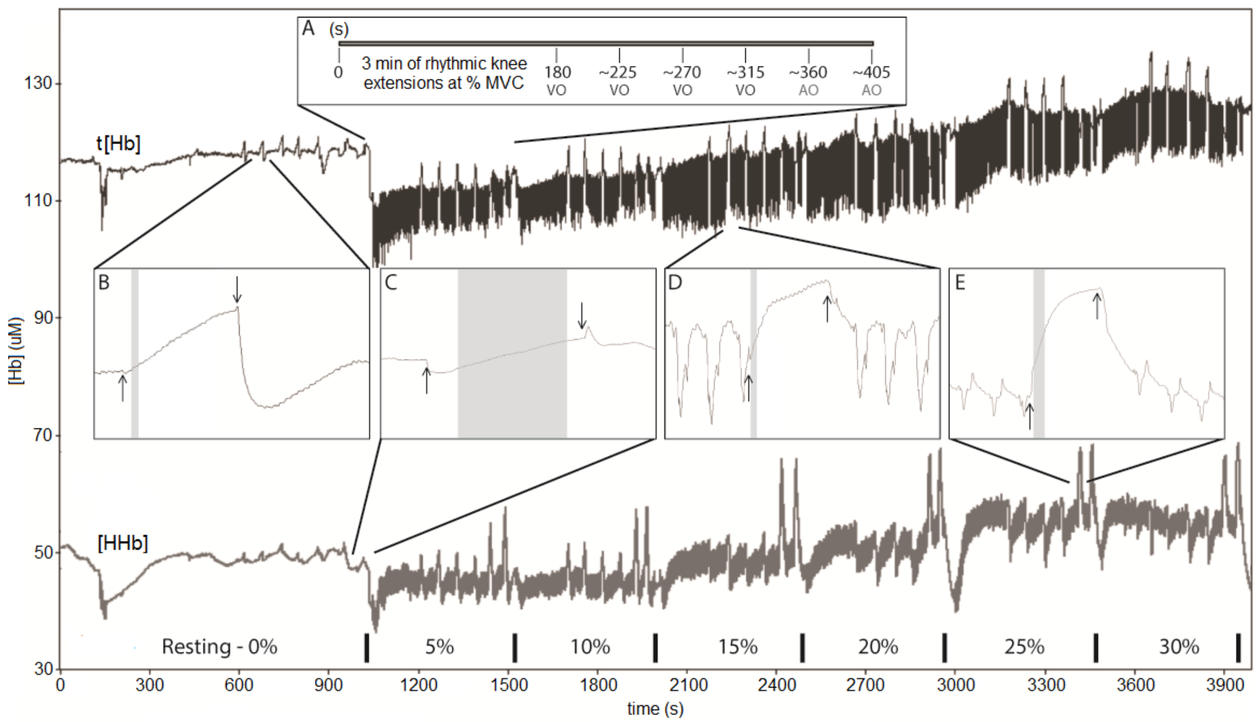

Figure 1 *Experimental Protocol.* Representative example of NIRS signals (µM) collected from visits 2-4 showing the raw [tHb] (dark grey) and [HHb] (light grey) traces. The horizontal black lines above the x-axis denote the start and end of each intensity level (%MVC). Panel A shows the timeline (s) for one exercise intensity (5% MVC). After 3 min of knee extension exercise the cuff was rapidly inflated for 5-10 s for 4 venous occlusions (VO; 70-80 mmHg) and 2 arterial occlusions (AO; 250-300 mmHg) with 45 s of knee-extension exercise between occlusions for the assessment of mBF and $m\dot{V}O_2$, respectively. Zoom panels B and C show [tHb] and [HHb] signals for VO and AO, respectively, during rest. Zoom panels D and E show [tHb] and [HHb] signals for VO and AO, respectively, during exercise with three knee extensions on either side of occlusion. Black arrows denote the inflation and deflation of occlusion and the shaded gray area denotes the linear increase used in the assessment of mBF or $m\dot{V}O_2$

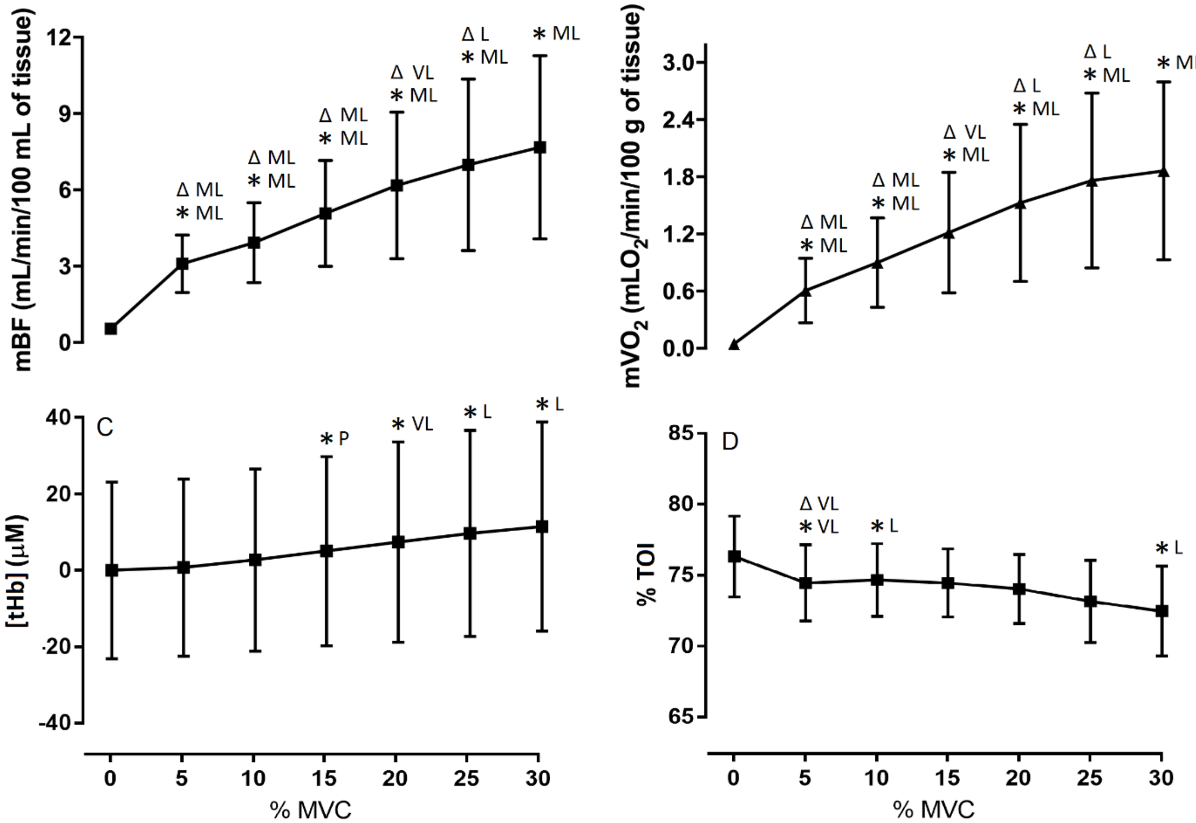

Figure 2 The responses of all NIRS parameters over all exercise intensities. to increasing exercise intensity. Panels show (A) mBF, (B) m$\dot{V}O_2$, (C) relative perfusion, and (D) TSI%. Data are means and bars standard deviation. Workloads substantially greater (smallest effect) than resting or the previous workload are denoted with an asterisk (*) or a triangle (Δ), respectively. Statistical likelihoods are given next to the symbol as possible (P, 50-74.9%), likely (L, 75-94.9%), very likely (VL, 95-99.49%) and most likely (ML, 99.5-100%)

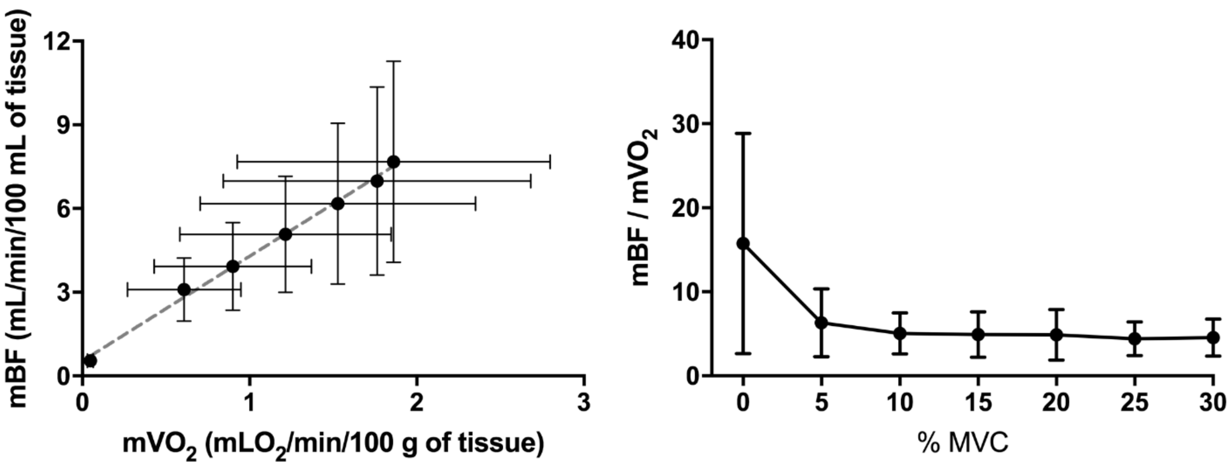

Figure 3 The relationship between m$\dot{V}O_2$ and mBF over all exercise intensities. (A) mBF as a function of m$\dot{V}O_2$ with the regression line denoted by the dashed grey line given by the equation $y = 8.071x + 0.5482$; $R^2 = 0.9914$. (B) m$\dot{V}O_2$/mBF ratio as a function of exercise intensity. Data are means and bars standard deviation

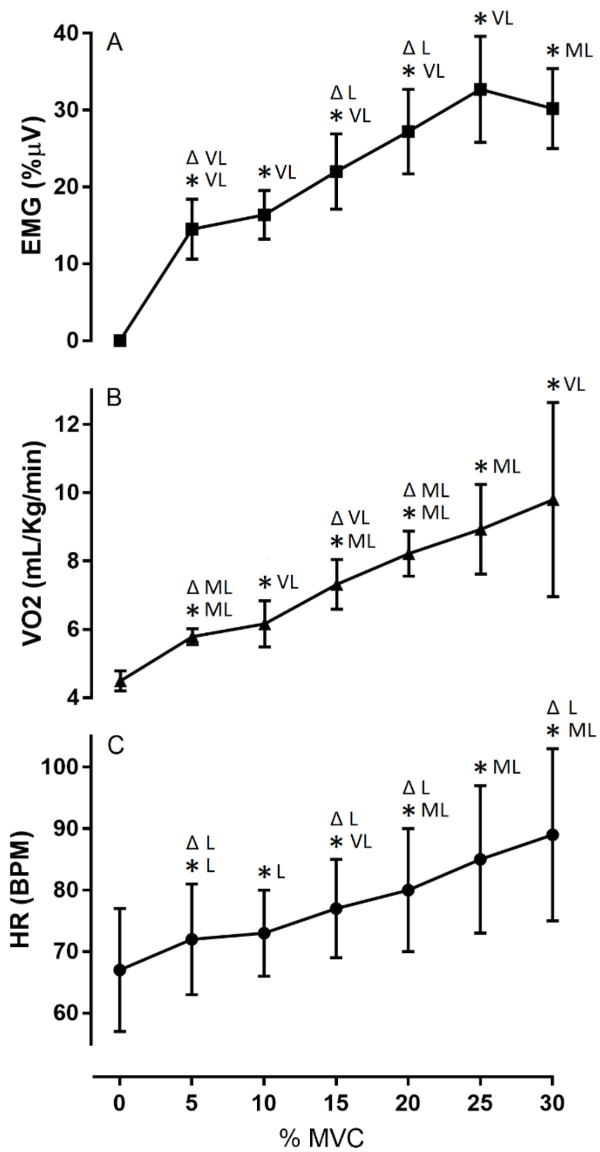

Figure 4 Responses of EMG (top), $\dot{V}O_2$ (middle), and HR (bottom) to increasing exercise intensity. Data are means and bars standard deviation. Workloads substantially greater (smallest effect) than resting or the previous workload are denoted with an asterisk (*) or a triangle (Δ), respectively. Chances are given next to symbol as possible (P, 50-74.9%), likely (L, 75-94.9%), very likely (VL, 95-99.49%) and most likely (ML, 99.5-100%)

Table 1 Mean values and standard deviations for participant characteristics.

	Age	Height (m)	Weight (kg)	ATT (cm)	VL (cm)	VL Belly (cm)
Male	27.0	1.8	75.0	0.4	3.1	1.98
SD	7.0	0.0	9.6	0.2	0.41	0.31
Female	21.00	1.67	61.40	0.57	2.99	2.07
SD	4.00	0.05	0.42	0.16	0.47	0.26

Abbreviations: ATT, adipose tissue thickness; VL, vastus lateralis; VL Belly, distance from skin to the belly of the VL calculated as ATT + 1/2*VL

Table 2 Reliability of mBF and $m\dot{V}O_2$ for rest and all exercise intensities.

	Rest	5%	10%	15%	20%	25%	30%
				mBF			
ICC	0.83	0.69	0.86	0.82	0.89	0.90	0.80
STE	0.45	0.59	0.41	0.46	0.37	0.35	0.48
CV (%)	14.6	31.0	21.4	24.8	20.9	20.2	30.4
SE (%)	5.5	7.7	8.9	9.0	10.0	10.2	10.1
				$m\dot{V}O_2$			
ICC	0.58	0.92	0.93	0.91	0.91	0.96	0.96
STE	0.68	0.31	0.30	0.34	0.33	0.22	0.22
CV (%)	50.4	20.5	20.0	22.6	24.2	14.0	13.5
SE (%)	9.2	12.3	12.1	12.0	13.3	12.2	11.9
				Relative Perfusion – [tHb]			
ICC	0.98	0.98	0.98	0.98	0.98	0.98	0.98
STE	0.18	0.16	0.17	0.17	0.15	0.17	0.16
CV (%)	4.0	3.7	4.0	4.1	3.8	4.1	3.8
SE (%)	4.5	4.6	4.6	4.7	4.9	4.9	4.9
				%TOI			
ICC	0.71	0.87	0.86	0.75	0.70	0.76	0.78
STE	0.57	0.40	0.41	0.53	0.58	0.53	0.51
CV (%)	2.4	1.6	1.5	2.1	2.3	2.4	2.6
SE (%)	0.7	0.7	0.7	0.6	0.6	0.8	0.9

Abbreviations: ICC, intra-class correlation coefficient; STE, standardized typical error. The STE magnitude thresholds are 0.1, 0.3, 0.6, 1, and 2 for small, moderate, large, very large, and extremely large; %CV, coefficient of variation; %SE, Percentage for smallest effect.

Additional Information: No competing interests or conflicts of interest exist with any authors. No external project fund was obtained.

<u>Supporting information:</u> Table 3. 90% Confidence limits for mean values of mBF, m$\dot{V}$O2, and tHb, and TOI% for rest & all exercise intensities.

Author Contributions:

A.A.L. and L.S. designed protocol with consult from J.F. and D.R.; A.A.L., G.A., W.L., and B.N., recruited and collected data; A.A.L. analyzed the data; A.A.L., D.C., D.R., and L.S. interpreted the data; A.A.L., G.A., W.L., B.N., drafted manuscript; A.A.L. prepared figures; A.A.L, D.C., J.F, D.R., and L.S. edited and revised manuscript; A.A.L, D.R., and L.S. approved the final version of manuscript.

Acknowledgements

We thank laboratory and other assistance from Shanggari Venugopal, Brooke Price, Brandon Woolley, Terrance Ryan, Kim Gaffney, Inge Ramakers, participants, and Roxanna J. Lucero.

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