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INTERPRETATION OF NEAR-INFRARED SPECTROSCOPY (NIRS) SIGNALS IN SKELETAL MUSCLE

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Abstract: NIRS uses the relative absorption of light at 850nm and 760nm, to determine skeletal muscle oxygen saturation. Previous studies have used the ratio of both signals to report muscle oxygen saturation. **Purpose:** To evaluate the different approaches used to represent muscle oxygen saturation, and to evaluate the pulsations of the O_2heme and Heme signal. **Method:** Twelve participants, ages 20-29years were tested on the forearm flexor muscles using continuous wave NIRS at rest. Measurements were taken during 2-3mins rest, during physiological calibration (5-minuts Ischemia) and during reperfusion. **Results:** There was a significant difference in pulse size between O_2heme and Heme signal at the three locations ($p < 0.05$). Resting oxygen saturation was 58.8+9.2%, 69.6+3.9%, and 89.2+6.9% when calibrated using O_2heme, TSI, and Heme, respectively. **Conclusion:** The difference in magnitude of O_2heme and Heme pulse with each heartbeat might suggest different anatomical locations of these signals, which propose calibrating with just one of the signals instead of the ratio of both. Calculations of physiological calibration must account for increased blood volume in the tissue, because of the changes in blood volume which appear to be primarily from the O_2heme signal. Resting oxygen levels calibrated with Heme agrees with theoretical oxygen saturation.

Keywords: Near Infrared Spectroscopy (NIRS), Oxygen consumption, Hemoglobin, Myoglobin, Skeletal muscle

1. Introduction

Near-infrared spectroscopy (NIRS) has been increasingly used to study oxygen levels in skeletal muscle [1-5]. A search of PubMed in January 2019 for the terms NIRS and muscle showed 679 publications with 126 from the last two years. NIRS measurements of muscle oxygen levels, blood flow, and metabolism are noninvasive and have shown good agreement with other measurement modalities [6]. Continuous wavelength NIRS devices uses physiological calibration (vascular occlusion) to 0%; this has been shown to allow accurate quantitative measurements in the skeletal muscle [7].

Several methodological questions about the NIRS measurements of skeletal muscle remain [5, 8], such as which of the NIRS signals is the most appropriate to report, and if physiological calibration (5-minute ischemia) reaches 0% oxygen saturation. NIRS measurements typically record absorbance of light at several wavelengths, where changes in absorbance in the region near 850nm is ascribed to oxygenated hemoglobin/myoglobin (O_2heme), and

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absorbance in the region near 760nm is attributed to deoxygenated hemoglobin/myoglobin (Heme). Often a ratio of absorbances at 850nm/760nm or 850nm/(850nm+760nm) are used to describe oxygen saturation [9]. A popular ratio is the Tissue Oxygenation/Saturation Index (TSI) which is the ratio of absorbance at 850nm/(850nm+760nm)x100 to produce values in percent[10]. However, some investigators such as Grassi et al. [5] has proposed using only the Heme signal because it seems to better reflect oxygen extraction in a variety of experimental approaches so that there are still questions on the most appropriate NIRS signal to report. There are also questions as to whether NIRS pulsation signal in skeletal muscle reflects contributions from hemoglobin or myoglobin[5, 11]. Some investigators have suggested that myoglobin represents 10% of the NIRS signal[12, 13], while others have reported it reflects 80% of the signal[14]. Davis et al. [8] reported approximately 32% hemoglobin in NIRS signal in human skeletal muscle; and that this value depends on anatomical and experimental position.

The purpose of this study was to evaluate the NIRS signals from 760nm and 850nm in response to ischemia and reperfusion in the forearms of healthy young adults. We hypothesized that: 1) Blood volume change alters the physiological calibration using the O_2Heme signal, producing a lower than expected oxygen saturation value, 2) The physiological calibration produced O_2heme values that can be considered zero oxygen saturation, and 3) pulsations in the NIRS signals due to heart rate reflects the oxygen saturation specifically from hemoglobin.

2. Materials and Methods

2.1 Participants. Twelve subjects (5 males, 7 females) aged 20-29yrs participated in the study. The study was conducted with the approval of the Institutional Review Board at the University of Georgia (Athens, GA) and the participants signed a written informed consent before testing.

2.2 Experimental design. This study used a single group design. Comparisons were made between three different experimental conditions (Rest, Ischemia and Reperfusion) performed during one testing session. The protocol consisted of comparing muscle oxygen consumption at rest, during 5 minutes of ischemia, and evaluating NIRS signals during reactive hyperemia after the release of ischemia.

2.3 Experimental Procedures. The measurement was done in the forearm flexor muscles with the subjects in a supine position. A continuous-wave NIRS device (Portamon, Artinis Medical Systems, Einsteinweg, Netherlands) was placed on the proximal/medial portion of the forearm [15], this was put in place with a non-elastic wrap. Adipose Tissue Thickness was measured using ultrasound (LOGIQ, GE Healthcare) as previously described [16]. NIRS measurements were digitally recorded throughout the protocol at an acquisition frequency of 10Hz.

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Neuromuscular electrical stimulation was used to increase muscle metabolic rate. Electrodes (2 x 2 cm) were placed proximally and distally to the NIRS device and connected to a commercial electrical stimulation device (RICH-MAR, theratouch 4.7, Version 15). The muscle was stimulated at 6Hz; Biphasic square wave pulses (200µs with a 50µs inter-pulse delay) with submaximal current levels (25-40mAmps) tolerable for each subject was used to activate the muscle to provide an increase in the metabolic rate. [15].

Muscle ischemia was produced with complete vascular occlusions with a blood pressure cuff, (Hokanson, Bellevue, WA) placed about 2cm above the elbow. The pressure of the cuff was set to 220–260 mmHg using a rapid cuff inflation system (Hokanson, E20 Rapid Cuff Inflator and a 30gallon capacity commercial air compressor).

2.4 Testing protocol. After a rest period of 2-3 minutes, blood pressure cuff was inflated for 30 seconds to measure the rate of muscle oxygen consumption, 2 minutes baseline measure was recorded. Electrical stimulation was introduced for 30secs to activate the muscle and check the rate of increase in muscle oxygen consumption; this helps identify the current level enough to activate each participants' muscle. Physiological calibration was performed (five minutes ischemia); cuff inflation for 5 minutes which was preceded by electrical stimulation using previously identified current to increase metabolic rate and reduce the time needed to reach full ischemia, electrical stimulation was also done 30 seconds to the end of the ischemia to check if oxygen consumption reaches 0%. Cuff was deflated, and measurement was taken until the signal reaches peak reactive hyperemia. Figure 1 shows the example graph of the protocol.

2.4 Data analysis. Oxygen consumption and oxygen delivery were measured using the oxygenated signal (O_2heme) and de-oxygenated signal (Heme) respectively. The raw data collected from the NIRS device was exported and analyzed on Microsoft Excel. Graph of the NIRS signal (Optical Density) was plotted against time (calculated using the frequency of data collection). The measurement was recorded from the third channel of the NIRS signal; this was done to avoid the influence of adipose tissue thickness at the shallow channel[17]. Optical density (O.D) was recorded at different points of oxygen consumption. The percent pulse size from each NIRS signal was measured and compared at rest, immediately after the ischemia (early reperfusion) and during peak reactive hyperemia. Pulse size was calculated by finding an average of three consecutive pulsations (wave heights); by subtracting the average of the two minimum pulse signal (troughs) from the maximum pulse signal (Crest), multiplied by 100; values are in optical density (O.D). All measurements were calibrated (physiological calibration) with the delta range of reactive hyperemia after the 5 minutes ischemia. Pulse size was also calculated as 32% percent of hemoglobin relative to total heme (hemoglobin plus myoglobin), as recommended by Davis and Barstow [8].

2.5 Statistical analysis. Data were analyzed using IBM SPSS Statistics software v24 (IBM®, Armonk, NY). One-way ANOVA was used to identify the difference among values of resting oxygen saturation calculated using O_2heme, Heme, and TSI signals, a pairwise

Bonferroni posthoc comparison was made to evaluate the individual paired difference. Paired sample t-test was used to test the difference in oxygen saturation before and after electrical stimulation during the physiological calibration (5mins cuff). 3x2 factor ANOVA was used to identify the difference between O_2Heme and Heme pulse size at rest, immediately after ischemia and during peak reactive hyperemia. Significance was accepted at $p \leq 0.05$ for all comparisons.

3. Results

Characteristics of the participants in this study are shown in Table 1. An example protocol used in this study is shown in Figure 1.

3.1. Oxygen Saturation

Figure 2a shows reactive hyperemia using the Heme signal and O_2Heme signal, including the difference in both signal (blood volume). There was a significant increase in blood volume during reperfusion, which was about 16% ± 6% in O_2heme signal. Resting oxygen saturation was calculated using three different methods (Figure 2b). There was a significant difference among the three values $F(2,27)=48.2$, $P<0.001$). Pairwise comparisons showed significant individual paired difference between the O_2heme signal, the TSI signal, and the Heme signal ($p < 0.05$ for all comparisons).

3.2. Physiological Calibration

With ischemia, the O_2Heme signal decreased, and the Heme signal increased, showing an increase in the deoxygenated blood and decrease in oxygenated blood due to muscle oxygen consumption, there were no significant changes in either signal with electrical stimulation after 4-5 minutes of ischemia (O_2Heme signal, $p=0.148$, and Heme signal, $p=0.598$). Figure 3 shows the mean difference and confidence interval of oxygen level before and after stimulation during the 5mins ischemia. TSI reaches approximately minimum 45% ± 11% and Maximum 76% ± 5% respectively.

3.3. NIRS Signal Pulsation

Representative image of heart rate induced pulse sizes from both O_2heme and Heme signals are shown in Figure 4. Pulse sizes were calibrated to the ischemic/hyperemia range and the assumed percent hemoglobin in the NIRS signals[8]. There is a significant difference between O_2heme and Heme pulse size ($F(1,54 = 113.8, \eta^2 = 0.68\ p < 0.001$). There was also a significant difference among the pulse size at the three locations (rest, cuff end and hyperemia)($F(2,54) = 43.0, \eta^2 = 0.61\ p < 0.001$). Pulse sizes were larger for the O_2heme signal compared to the heme signal and was largest later during reactive hyperemia compared to during early reperfusion and when at rest (Figure 5a) The ratio of pulse sizes between O_2heme and Heme were not similar to the oxygen saturation values for the entire NIRS signals (Figure 5b).

Figures, Tables, and Schemes

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Table 1. *Characteristics of Study Participants*

	Male	**Female**
N	5	5
Age (yrs)	23.6 (4.3)	20.2 (0.25)
Height (cm)	170 (0.2)	1.66 (0.04)
Weight (Kg)	73.4 (12.3)	64.22 (10.5)
Body Mass Index (Kg/M^2)	25.2 (3.4)	23.51 (4.9)
Adipose tissue Thickness (cm)	0.34 (0.09)	0.40 (0.22)

Values are means (standard deviations)

Figure 1

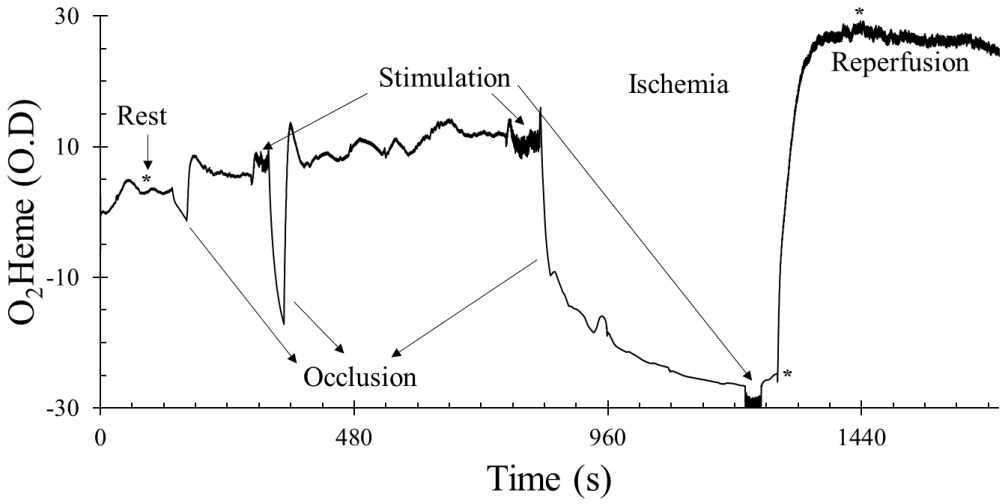

Figure 1. An example of the testing protocol using O_2heme signal. The y-axis scale is in optical density units. * shows the approximate time points when pulse size was calculated at rest, immediately after ischemia and during reactive hyperemia. O_2heme and Heme physiological calibration reaches 0% during ischemia and 100% at peak hyperemia,

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Figure 2

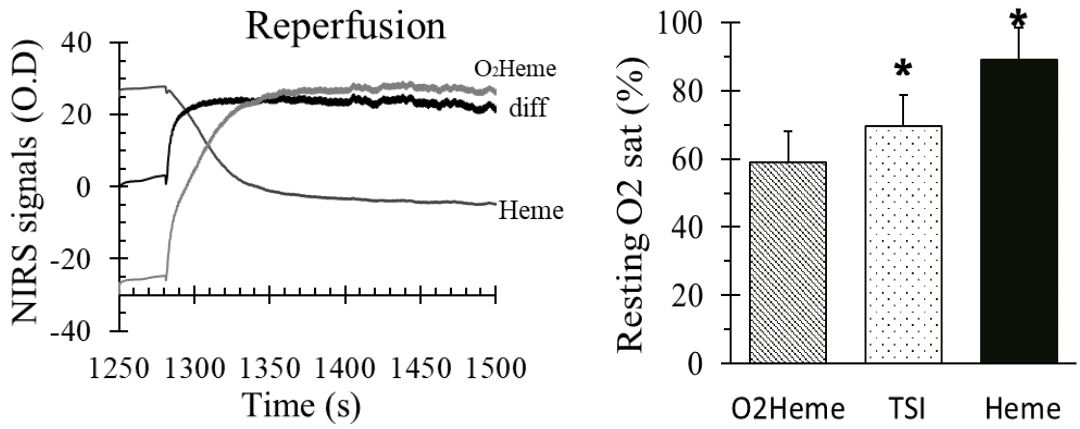

Figure 2. **A)** The O_2heme and Heme signal during reactive hyperemia and the difference in both signal which indicated the influence of blood volume change. **B)** The percent of oxygen saturation at rest from three different methods of calculating oxygen saturation (O_2heme, TSI, and Heme). *Indicates a significant difference in the method of calculating oxygen saturation ($P < 0.01$ for the three comparisons). Values are means ± SD.

Figure 3

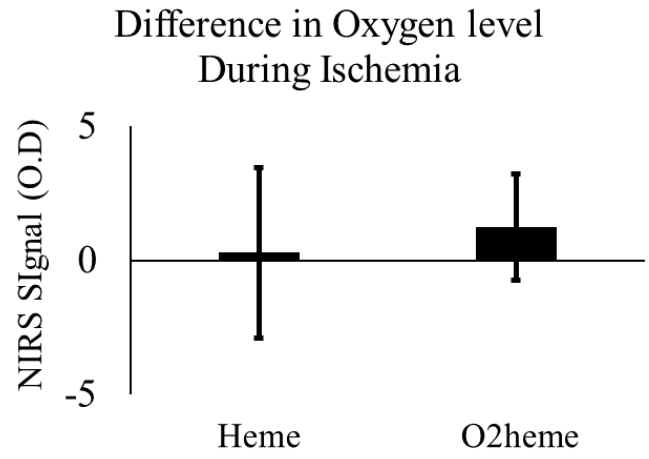

Figure 3. The change in O_2heme and Heme signals before and after stimulation during the 5 minutes of ischemia with prior stimulation. There was no significant difference, $P > 0.05$. Values are means with the 95% confidence intervals.

Figure 4

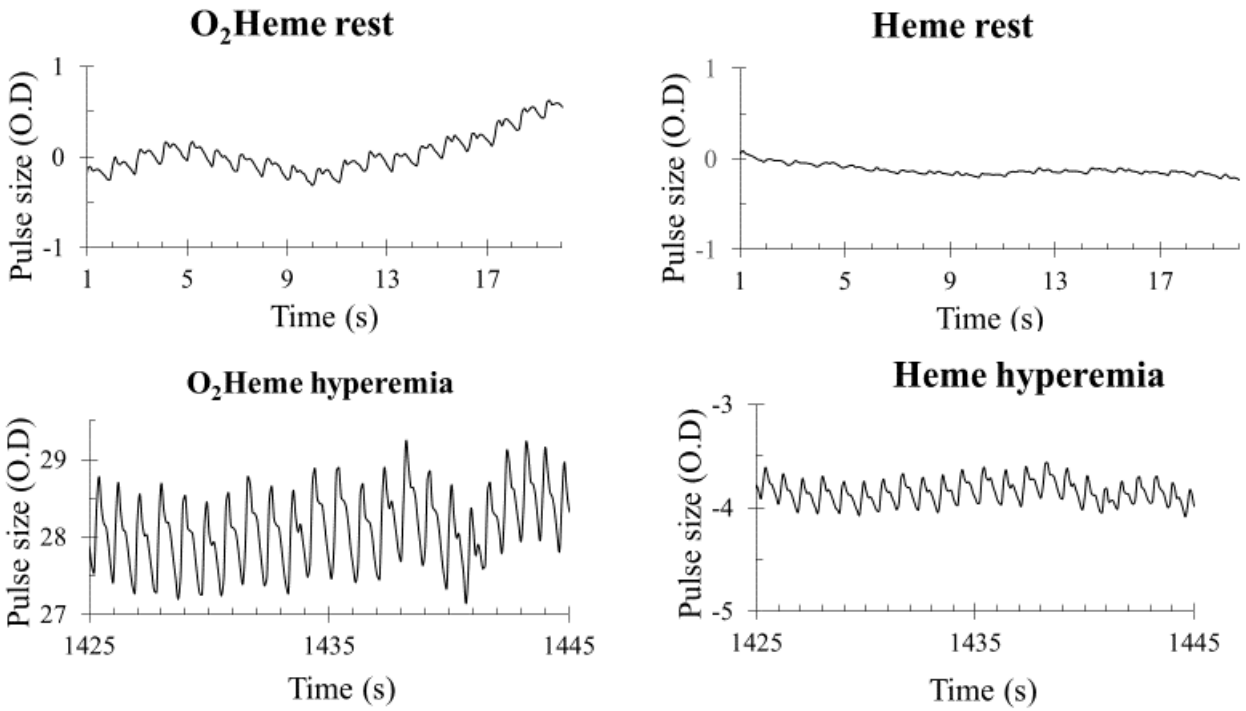

Figure 4 Examples of blood flow pulsations of O_2heme and Heme signals at rest and during peak reactive hyperemia. The y-axis scale is in optical density units.

Figure 5

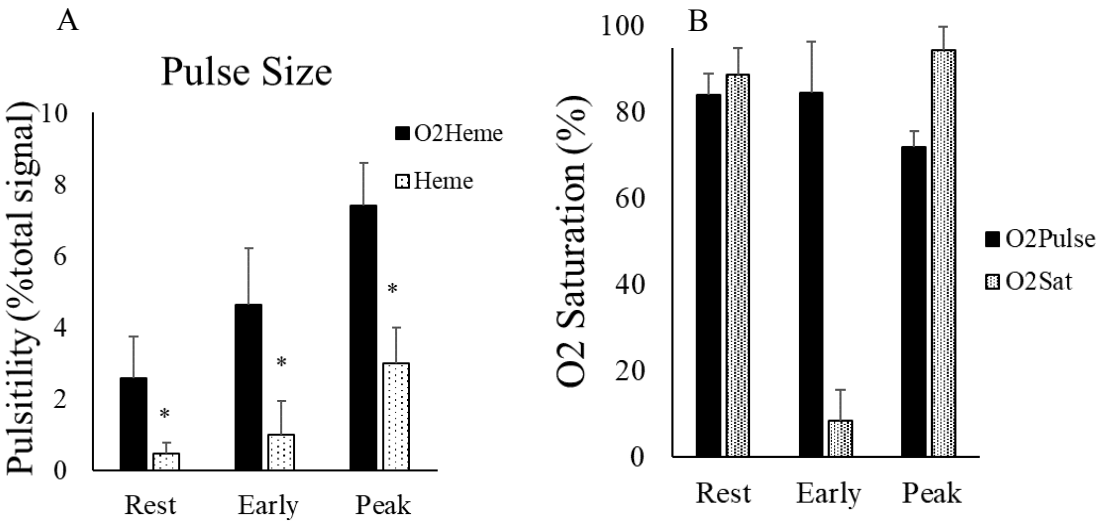

Figure 5. **A)** The average pulse size signals of O_2heme and Heme at rest, immediately after ischemia, and during reactive hyperemia**.** The y-axis scales are in % of the calculated physiological calibration. *indicates a significant difference in the pulse size at each location. #indicates a significant difference between the O_2heme and Heme signal. P <.001 for all comparisons. **B)** The ratio of the pulse size at the three difference locations and the oxygen saturation calibrated to each signal at the same location. Values are means ± SD.

4. Discussion

This study evaluated the use of NIRS signals to determine oxygen levels in skeletal muscle. NIRS signals from 850 and 760 nm light were presented as O_2heme and Heme to reflect the contribution of myoglobin and hemoglobin. Previous studies have presented NIRS signals as O_2Hb and HHb, or as Hb/Mb[1-5]. This was done based on observations that the NIRS signals come from both hemoglobin and myoglobin, and the need to provide a more precise terminology for NIRS signals [18].

4.1. Appropriate NIRS signal for oxygen saturation in the muscle. This study found that the physiological calibration includes a transient increase in blood volume, which appears to be entirely from the O_2heme signal. Calculating a resting oxygen saturation value using O_2heme or ratio of both O_2heme and Heme (such in TSI) resulted in oxygen saturation values that were lower than values obtained if only Heme signal is used. The resting oxygen saturation values (~59%) found with the O_2Heme difference signal was similar to that seen in previous studies (<70%) [19, 20]. However, using only the Heme signal for the physiological calibration results in resting oxygen saturation values of 91%. This value is consistent with the expected value of Heme oxygen saturation (~88%) based on the assumptions that: at rest myoglobin oxygen saturation is 100% [21], the myoglobin contribution to the total Heme in muscle is 70%, and hemoglobin saturation values of 70% (between 98% in the artery and 40% in the veins) and a hemoglobin contribution to the total Heme of 30%. For the resting oxygen saturation of the total Heme to be near 60%, either there must be significant myoglobin desaturation at rest, or hemoglobin oxygen saturation needs to be less than zero, neither of which is supported in the literature. The use of deoxygenated signal (Heme) to reflect changes in oxygen levels in muscle has been suggested previously[5, 22, 23]. Interestingly, TSI is a commonly used approach to present oxygen saturation values, and in our study resting TSI was 70%, consistent with previous studies. TSI also does not agree with the calculated Heme oxygen saturations (above). TSI is determined from the ratio of light absorbance at the two wavelengths and never approaches either zero or 100% oxygen levels during a physiological calibration. Therefore, we propose that oxygen saturation should be calibrated with the (physiological calibration) ischemia protocol [24] using the Heme signal.

4.2. Physiological calibration using 5mins ischemia with prior exercise. This study found that 5 minutes of ischemia with prior 30 seconds of electrical stimulation resulted in a minimal value of heme oxygen levels. This was shown by the lack of change in either the O_2heme or the Heme signals after an additional electrical stimulation once the signals had plateaued. Previous studies have not always found that 5 minutes of ischemia produced minimal oxygen levels or maximal reactive hyperemia. Five minutes of ischemia has produced 80-90% change in oxygen saturation levels[25] and about 80-90% of the maximal hyperemic blood flow response measured with ultrasound[26]. However, the use of prior exercise to increase metabolic rate (in this study produced an increase in metabolic rate approximately 5-fold above resting metabolic rate) did appear to result in complete

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desaturation of the muscle. The prior use of exercise or electrical stimulation to increase metabolic rate have been proposed to assure a maximal change in oxygen levels and blood flow[27].

4.3. Interpretation of the pulsitile O_2heme and Heme signals from muscle. We could not accept the hypothesis that pulsations in the NIRS signals reflect the oxygen saturation from entire hemoglobin signal. Because the pulsations are due to changes in the hemoglobin signal and not the myoglobin signal, the hypothesis was that the ratio of signal size of the O_2heme and Heme pulsations would reflect hemoglobin oxygen saturation. However, the ratio of signal size of the O_2heme and Heme pulsations were significantly different from the hemoglobin oxygen saturation values that would be expected during imediate reperfusion at low oxygen levels as well as during reactive hyperemia once oxygen levels were maximal. Pulsations blood due to beating of the heart are thought to disappear in capillaries and venules[28]; however more recent studies have found evidence of pulsation in skeletal muscle capillaries [29]. An alternative hypothesis is that the O_2heme and Heme pulsations represent oxygen saturation levels on the arterial side of the micovascular system. The size of the pulses relative to estimated total hemoglobin concentrations is small, although this increases during reactive hyperemia when vascular tone is decreased. The presence of pulsations in the heme signal during peak reactive hyperemia suggest that the 100% value for total heme from the physiological calibration is actually less than 100%. This is to be expected as the muscle is still consuming oxygen even during the peak reactive hyperemia time period. However, the size of the Heme pulsations are small enough to allow the assumption of 100% to be close to accurate, and it would be difficult to accurately determine how much different the actual value would be from 100%. Thus, the pulsations in the O_2heme and Heme signals from NIRS most likely come from precapillary arterioles[30], although changes in vascular tone might alter the micorvascular area that contibutes to the signal[31].

4.4 Limitations. This study was performed using one type of NIRS device; the continuous wavelength 'Portamon' from Artinis Ltd. Most continuous wavelength devices use similar wavelengths and calculations to determine O_2heme and Heme signals. While the results might be expected to be similar for other continuous wavelength devices, other devices use phase modulation of photon counting to determine both absorption and scattering of light, allowing more accurate calculations of oxygen levels. How these devices determine O_2heme and Heme might be different enough to produce different relationships between the variables. We used an assumption of the relative contribution of myoglobin and hemoglobin in human muscle based on Davis and Barstow [8]. This assumption was used as a general approximation as hemoglobin levels can vary between people and between experimental conditions[8]. We also tested our subjects in a supine position and did not alter body position during the experiment. Studies where the body is in the standing position, where there are changes in body position, or where the muscle of interest is at a different height than the heart might have different changes in blood volume from our finding. Under those

circumstances, the changes in blood volume might not reflect purely O_2heme as they did in our study.

5. Conclusions

This study found that a physiological calibration using ischemia with prior exercise can determine a range of oxygen levels in muscle that goes from 0%-100%. Calculations of a physiological calibration must account for increased blood volume in the tissue due to reactive hyperemia. Because of the changes in blood volume, which appear to be primarily from the O_2heme signal, the Heme signal is perhaps a better signal to perform the calibration with. Finally, NIRS based signals have heart rate related pulsations in signal intensity, which most likely reflect hemoglobin in the atrial side of the microvascular system and not the entire hemoglobin signal.

Author Contributions:

Conceptualization, Kevin McCully; methodology, Adeola Sanni, Kevin McCully; software, Kevin McCully; formal analysis, Adeola Sanni; investigation, Adeola Sanni; data curation, Adeola Sanni; writing—original draft preparation, Adeola Sanni; writing—review and editing, Adeola Sanni, Kevin McCully; supervision, Adeola Sanni, Kevin McCully; project administration, Kevin McCully.

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Conflicts of Interest: One of the authors; Kevin McCully is the President and Chief Science Officer of Infrared Rx, Inc, a company that develops analysis software related to NIRS measurements.

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