Article

EFFECTIVENESS OF 6-ISCHEMIC CUFF NEAR INFRARED SPECTROSCOPY MITOCHONDRIAL CAPACTITY TEST

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Abstract: Near-Infrared Spectroscopy (NIRS) has been used to measure muscle mitochondrial capacity. The current method requires as many as 22 short ischemic occlusions to generate a recovery curve for mitochondrial capacity. PURPOSE: To determine the effectiveness of using a 6-occlusion analysis protocol to study muscle mitochondrial capacity. METHOD: Two independent, unidentified data sets were analyzed (bicep n=48, forearm n=41) from previous studies using a NIRS device (Artinis, Ltd.). Both data sets had two recovery tests that included 22 ischemic occlusions. A recovery rate used to indicate mitochondrial capacity was calculated two different ways (simultaneously). Each sample was analyzed with a MATLAB program; with a curve-fit for the 22 ischemic occlusions and curve matching for the first six ischemic cuffs and an end resting value. The two resulting rate constants were compared using correlations, both for the two data sets, good and bad fitting data, using the best 5 of 6 points for the 6 cuff approach. RESULTS: The rate constants were not significantly different between the 22 cmuff and 6 cuff for the total data sets: bicep ($1.43\pm0.32 min^{-1}$, $1.44\pm0.35 min^{-1}$, p=0.56), forearm ($1.94\pm0.42 min^{-1}$, $1.95\pm0.44 min^{-1}$, p=0.76). The average bicep rate constants, when compared to each other, had an equation of y=1.07x-0.09, R^2=0.90. The average forearm rate constants, when compared to each other, had an equation of 0.98x+0.02, R^2=0.93. CONCLUSIONS: The 6-Cuff analysis provided the same results as the longer 22-cuff. The 6-cuff approach is both shorter in time and uses less ischemic occlusion periods, increasing the practicality of the NIRS mitochondrial capacity test.

Keywords: Near Infrared spectroscopy; NIRS; Skeletal muscle; muscle metabolism; electrical stimulation

1. Introduction

Near Infrared Spectroscopy (NIRS) has been used in previous studies as a non-invasive approach to measuring muscle oxygen consumption as a gauge of mitochondrial capacity [1,2] as well as skeletal muscle blood flow [3-5]. It has been used to study muscle mitochondrial capacity in clinical populations [6], as well as endurance athletes [7]. Furthermore, muscle mitochondrial capacity been characterized in multiple specific disease pathologies such as those with spinal cord injuries [8], cystic fibrosis [9], multiple sclerosis [10], and amyotrophic lateral sclerosis [11]. Several review papers have been written on this subject [6,12].

Two important limiting factors to measure muscle mitochondrial capacity using NIRS exist. One issue is the need to use repeated arterial occlusions after exercise[2]. These occlusions are needed to obtain a good fit to an exponential curve, the rate constant of which being the index of mitochondrial capacity. In order to accurately fit to an exponential curve, 18 – 22 ischemic blood occlusions are typically used to accurately produce a mono-exponential curve to a steady baseline. The issue with this method is the large number of ischemic cuffs required to obtain an accurate measurement. If two tests are performed, a participant must undergo a minimum of forty-four ischemic cuffs (twenty-two

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ischemic cuffs for each of two individual mitochondrial capacity test). This large number of cuffs can be difficult to tolerate, especially in at-risk and elderly populations. A second limiting factor is the requirement for a blood volume correction factor [2] in order to correct for changes in light absorption when the ischemic cuff inflates. Recent studies have found the blood volume correction does not completely correct for changes in light absorption when the ischemic cuff inflates. This incomplete correction is seen especially when measuring lower mitochondrial rates near the end of recovery. A test of muscle mitochondrial capacity could be improved if a method to address these limitations were developed.

Prior studies of muscle mitochondria capacity have fit data to an exponential curve function. Based on this prior knowledge, it can be assumed that muscle mitochondrial capacity follows an exponential curve function and therefore data can be matched to an exponential curve function rather than created new for each protocol. The aim of this study is to use the first few ischemic cuffs of a mitochondrial test to obtain curve fits that match the curve fits from the entire set of cuffs. Data will be obtained from previous studies. It is hypothesized that the abbreviated test protocol (Mito6) will produce the same results as the currently employed mitochondrial test protocol (Mito22).

2. Materials and Methods

2.1. Participants.

This study made use of two independent unidentified data sets (bicep n=48, forearm n=39). Subject characteristics are shown in Table 1. Both studies were conducted with approval of the Institutional Review Board at the University of Georgia (Athens, Ga), and all of the subjects were gave written, informed consent before testing.

2.2. Experimental design.

Two de-identified data sets were collected using a standard NIRS mitochondrial capacity test and had a full set of 22 ischemic cuffs for each test in the method as described in T.E. Ryan, et al., 2012[13]. This complete set of data underwent two separate analysis approaches and was analyzed separately in order to reduce bias.

2.3. Experimental Procedures.

While the data was the same, the number of actual data points used in each of these analysis protocols differed between approaches. The separate approaches are described below.

Approach 1 – Mito22: A standard mitochondrial capacity analysis [14] was completed on the de-identified data sets in order to measure the rate of recovery of muscle oxygen consumption, representing mitochondrial oxidative capacity. A representative example of this data used can be seen in Figure 1. The rate of recovery of muscle oxygen metabolism was quantified by fitting the oxygen consumption rates to the exponential equation y(t)=End-Δ×e^(-kt). The rate constant k, was used as an index of muscle oxidative capacity.

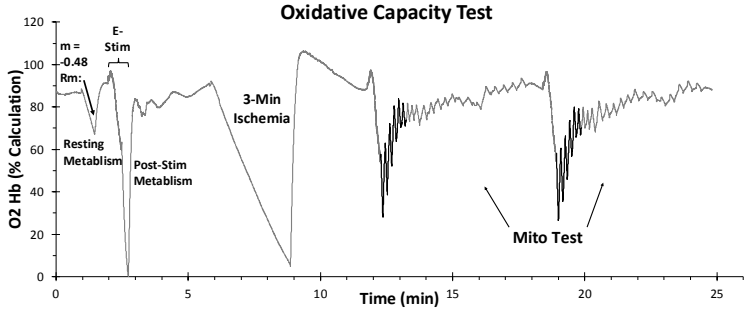

Figure 1. Representative example of the full protocol for the mitochondrial capacity test, which includes resting metabolism, post stimulation metabolism, 3-minute ischemia, followed by two mitochondrial capacity tests.

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Approach 2 – Mito6: The proposed mitochondrial capacity test analysis was also used on the de-identified data sets. This analysis only used the first six ischemic cuff slopes and a resting value in order match to a mono-exponential curve to a baseline. The first six points were systematically compared to exponential curves that used the first point and the end resting point, but with varying rate constants. The rate constant from the curve with the lowest combined residual for the six points was selected as the mitochondrial capacity rate constant. Systematically throwing out the point with the highest residual value and refitting was also attempted. This method changed the output values but did not make the fit any better. The fits of the good data and bad data were not significantly different from the original combined data set and therefore implies that the Mito6 protocol is less prone to error due to bad data.

A representative example of the slope of oxygen consumption for the six and 22 cuff approaches are shown in Figure 3.

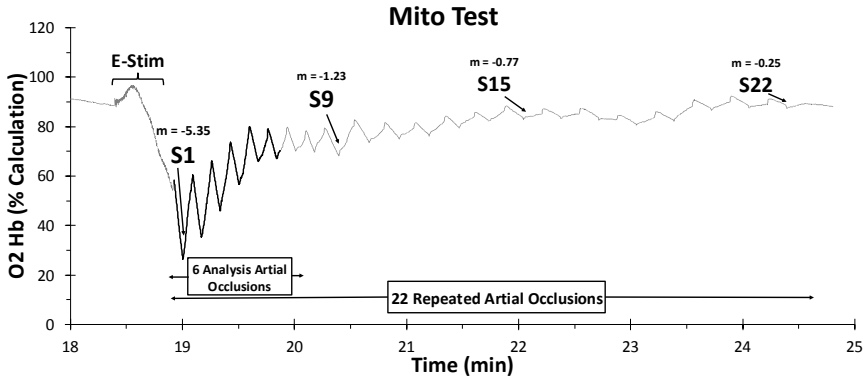

Figure 2. Example of the zoomed in O_2 Hb signal during exercise and arterial occlusions for a full 22-Cuff Test. Slopes become less steep over time, illustrating the recovery of O_2 consumption after exercise.

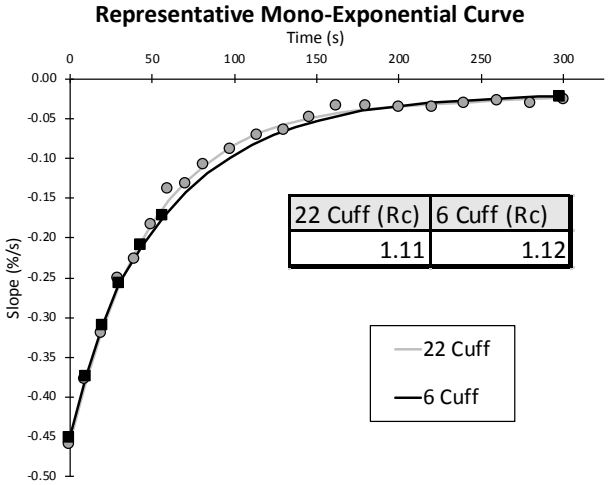

Figure 3. Representative input example into the custom MatLab program. Representative mono-exponential curve fitted to the measurements of oxidative consumption. The resulting rate constant is directly proportional to mitochondrial capacity. The black mono-exponential curve was used in the calculation of the 6-Cuff measurement and only used the first 6 ischemic cuffs and a final resting cuff. The grey mono-exponential curve was used in the calculation of the 22-Cuff measurement and all available points were used to fit the mono-exponential curve.

2.4. *Data analysis.*

The NIRS tests were analyzed using custom-written routines in MatLab v. 9.2.0.556344 (Mathworks, Natick, MA). Simultaneously, in both these protocols, slopes were identified, blood

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volume correction was applied, and the measured resulting slopes were fit to a single mono-exponential curve to a steady baseline. The Mito22 approach used all 22 measured slopes while the Mito6 approach used only the first six slopes as well as an endpoint value to fit/match these curves and produce appropriate rate constants.

2.5. Analysis of Approaches.

The measured rate constants of both approaches were compared through regression analysis, of all iterations completed. Once multiple variables were defined and controlled for, the final Mito22 and Mito6 analysis protocols of averaged multiple trials were compared to determine accuracy and usability. Furthermore, percent difference values were calculated and graphed for each iteration. These were used in order to determine if any systematic bias was present.

3. Results

Characteristics of the participants in this study are shown in Table 1.

Table 1. Subject characteristics from the two data sets.

	Bicep			Forearm		
	Male	Female	Overall	Male	Female	Overall
Number (n)	2	5	7	10	13	23
Age (yrs)	20.00±0.00	20.00±0.00	20.00±0.00	27.95±6.18	22.62±3.48	24.94±5.43
Height (cm)	171.45±8.98	168.15±11.56	169.09±10.25	180.23±5.69	164.98±6.61	171.61±9.84
Mass (kg)	65.09±9.30	61.14±8.83	66.13±18.10	86±10.82	64.48±7.02	73.83±13.92
Total Samples(n)	13	35	48	17	22	39

Values are means (standard deviations).

3.1. Rate Constant Comparison

The overall process and specific iterations of developing the 6-Cuff Mitochondrial capacity test can be seen in Figure 4 for the bicep data. Figure 4a shows an analysis of the two protocols when the endpoint value was controlled for (i.e. made the same for both analysis protocols) (R2 = 0.84, m = 1.00). The data was then separated into two different categories – "good fit" and "bad fit," – based on the arbitrary value discussed above. These characterizations allow for specific conclusions to be made in the different data categories. Figure 4b represents the linear regression of the "Good Fit" data (R2 = 0.85, m = 1.05). Figure 4c represents the linear regression of the "Bad Fit" data (R2 = 0.82, m = 0.99). Overall, there was no true effect on the fitting of the exponential curve between the "Good Fit" and "Bad Fit" data.

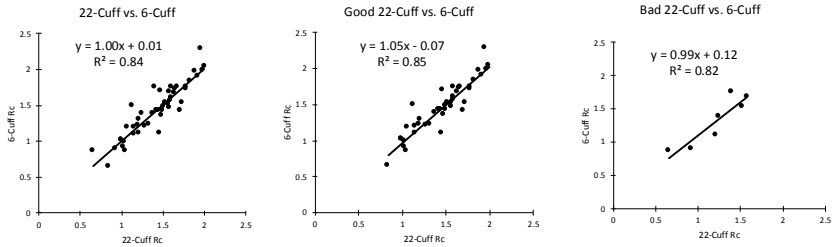

Figure 4. A) Comparison of two analysis protocols. 22-Cuff measurement rate constants versus 6-Cuff measurements. All points included in measurements and comparisons. Shows comparison of two analysis protocols when the same endpoints for measurements were used. **B)** Comparison of two analysis protocols. 22-Cuff measurement rate constants versus 6-Cuff measurements. Good fit data points included in measurements and comparisons. Shows comparison of two analysis protocols when the same endpoints for measurements were used and bad fit data was excluded. **C)** Comparison of two analysis protocols. 22-Cuff measurement rate constants versus 6-Cuff measurements. Bad fit data points included in measurements and comparisons. Shows comparison of two analysis protocols when the same endpoints for measurements were used and good fit data was excluded.

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Determination of the rate constant of these separated data sets when the point of highest residual was removed was also attempted. By attempting to systematically throw out the point with the highest residual value and refitting, it was hypothesized to decrease the error present in the analysis protocols. While this method changed the output values, it did not make the fit any better and caused the output to have a higher variance. The net result being no advantage to the fitting of the data to the mono-exponential curve, so this method was discontinued.

The individual data sets were then paired with their specific trials and averaged in order to create a multiple trial average of the data for the 22-Cuff Rate Constants compared to 6-Cuff Rate Constants. In normal use of NIRS analysis, it is normal to take multiple trials under each specific condition and average them together. This characterization can be seen in Figure 5a (R2 = 0.90, m = 0.1.07) for the bicep multiple trials averages and Figure 5b (R2 = 0.93, m = 0.98) for the forearm multiple trials averages . None of the correlations showed evidence of systematic bias as analyzed with the percent difference plots plot, as seen in Figure 5c and 5d.

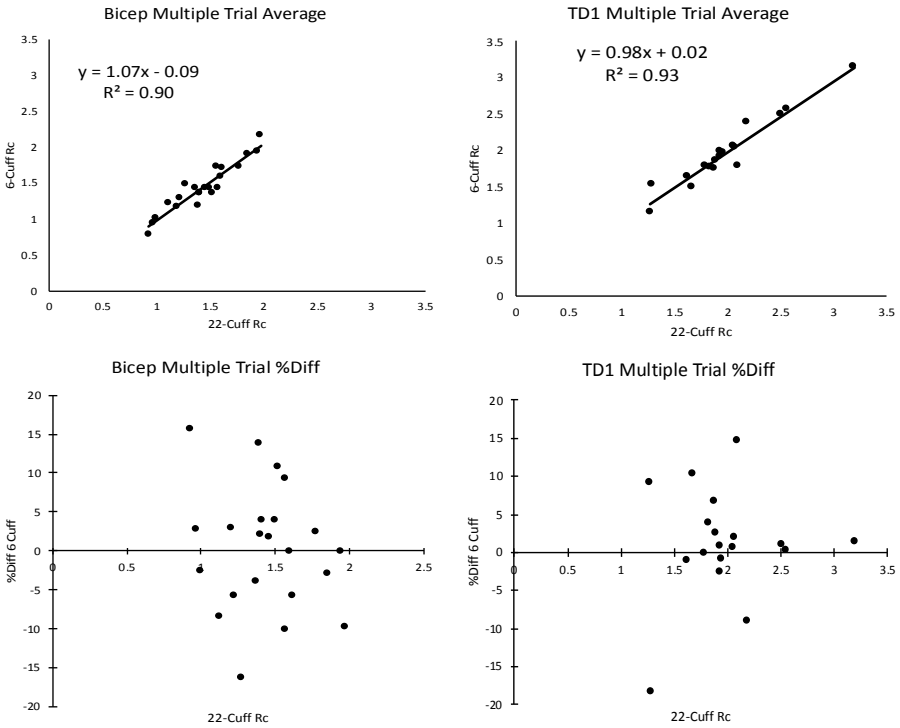

Figure 5. A) Comparison of two analysis protocols with multiple trials averaged together using bicep muscle data. 22-Cuff measurement rate constants versus 6-Cuff measurements. **B)** Comparison of two analysis protocols with multiple trials averaged together using forearm muscle data. 22-Cuff measurement rate constants versus 6-Cuff measurements. **C)** Percent difference of the average of analysis protocols with multiple trials averaged together using bicep muscle data. 22-Cuff measurement rate constants versus 6-Cuff measurements. **D)** Percent difference of the average of analysis protocols with multiple trials averaged together using forearm muscle data. 22-Cuff measurement rate constants versus 6-Cuff measurements.

4. Discussion

The primary finding of this study was that rate constant of recovery of metabolic rate after exercise was not different when calculated from a reduced number of data points (Mito6) compared to a full set of data points (Mito22). Previous studies have measured muscle mitochondrial capacity using exercise to rest transitions and curve fitting similar to the Mito22 used in this study[6,12]. Collecting data points throughout the entire recovery process can result in accurate curve fits, as indicated by coefficients of variation between 8-12% [13] [14]. However, a limitation of this approach is that 18-24 short ischemic periods are needed to perform curve fitting. The Mito6 protocol only

requires 7 ischemic periods (one additional point for the full recovery time point). Thus the Mito6 protocol significantly reduces the number of ischemic periods needed, especially if 2 or 3 experiments are performed to increase the accuracy of the measurement [8,15].

A second benefit of the Mito6 protocol is a reduced reliance on data points with low metabolic rates (later in the recovery process). With continuous wavelength NIRS devices as used in this study, inflating a blood pressure cuff can change the scattering of light which is not directly detected by the continuous wavelength NIRS device [16]. The changes in scattering appear to be similar in magnitude on the absorption signal to the changes in absorption of resting metabolism [13] [17]. Because of this, corrections for scattering changes ("blood volume correction") must be very accurate or there will be errors in correcting these data points, which will influence fitting the exponential curve. During the early points in recovery, the metabolic rate is much higher, and thus the influence of the correction factor is less. The Mito6 protocol thus has the advantage of not being as dependent on accurate corrections for changes in scattering.

A critical factor in the Mito6 approach is the use of a final recovery point to find the best exponential curve. This study found that the best outcomes were obtained when a post test recovery value was used, rather than using an initial resting metabolism point. This was based on getting closer agreements with the Mito22 and Mito6 analysis procedures. Because the post test recovery value was higher than the initial resting value, this suggest that during the recovery tests muscle metabolism does not completely recover to resting values. Even though using a post recovery metabolic rate value requires an additional 5 minutes added to the protocol, the results suggest that post-exercise oxygen consumption by the muscle is still present at five minutes, and needs to be accounted for with the analysis program.

This study chose to measure 6 initial recovery points to find the rate constant of the exponential curve. This seems appropriate for the rate constants found in the two studies that were evaluated (approximately 1.5 minute-1). Studies of endurance trained athletes or people with reduced mitochondrial capacity may benefit from a different number of initial data points. In our study we did evaluate the potential value of using the best 5 initial data points based on reducing the residuals of the curve fit, rather than all 6 of the initial points. Because we found that across our two data samples we essentially got the same result, we don't feel this approach is necessary to optimize data analysis.

The determination of what was considered "good fit" and "bad fit" data, was decided arbitrarily. However, no matter what cut off point is determined, there will always be data that is considered "good fits" and "bad fits." This value allowed the researchers to systematically separate the data to determine how it acted within the analysis protocols.

A possible limitation to the use of the Mito6 approach is how well the method would fit data that had lower 'quality'. It is expected that all methods of curve fitting would work on higher quality data. In our study we found the Mito6 approach appeared to work equally well on lower quality data, as judged by having lower R^2 values for the fit with the Mito22 analysis. This suggests that the Mito6 approach can be used on data that has a range of quality. In general NIRS based recovery measurements of mitochondrial capacity have better curve fits that ^{31}P MRS fits of phosophcreatine recovery after exercise [18]. However, some study populations have greater adipose tissue thickness over the muscle of interest [8], and great adipose tissue can reduce the quality of the data for NIRS studies [19]. This study evaluated two data sets on relatively young and healthy subjects, one on the biceps muscle and the other on forearm muscles. Additional studies evaluating the Mito6 approach should be done on data sets where there is reduced signal and the quality of the data is less.

In conclusion, the Mito6 analysis protocol which uses the first few data points along with a recovery time point, can be used as an accurate alternative to the currently used Mito22 analysis protocol that includes data points throughout the full recovery period. An advantage of the Mito6 approach is that it takes less time and requires fewer ischemic measurement periods. Future studies of mitochondrial capacity using NIRS should consider using this approach.

4.1. Limitations.

Possible limitations to this study include the accuracy of the endpoint value which is measured, as this has a large effect on the curve matching equation. This limitation, however, can be controlled for by ensuring the participants are fully at a resting state when this value is taken. If the participant is fully at rest, muscle mitochondrial capacity is considered to be at equilibrium and this value can be confidently used in the determination of the endpoint in the curve matching equation.

Another possible limitation to this method includes its limited accuracy for data which is considered to be a bad data set. However, this data would not produce fully accurate rate constants with either analysis protocol as both programs would be susceptible to the issues with the data itself. Therefore, it can be considered that the 6-Cuff analysis protocol is better for this "bad fit" data as it decreases the protocol time and controls for outliers in a more efficient manner in comparison to the 22-Cuff analysis protocol.

5. Conclusions

The Mito6 analysis protocol can be used as an accurate alternative to the currently used Mito22 analysis protocol. Furthermore, this analysis protocol requires less time and less stress on participants and researchers alike. Lastly, the new Mito6 analysis protocol handles "bad data" better than the Mito22 analysis protocol as it has a mechanism to remove outliers and is less susceptible to issues with the blood volume correction factor.

Author Contributions: Conceptualization, Kevin McCully, Maxwell Sumner; methodology, Kevin McCully, Maxwell Sumner; software, Indrajit Das; formal analysis, Maxwell Sumner; investigation, Maxwell Sumner, Elizabeth K Pryor; data curation, Maxwell Sumner, Elizabeth K Pryor; writing—original draft preparation, Maxwell Sumner; writing—review and editing, Maxwell Sumner, Kevin McCully, Elizabeth K Pryor; supervision, Max Sumner, 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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