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William Durbin
University of Dayton

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Abstract

Reactive hyperemia tests create mismatches in oxygen (O2) delivery and demand by occluding blood flow and O2 delivery in the face of stable quiescent skeletal muscle O2 demand. We tested the hypothesis that skeletal muscle reactive hyperemia is dependent on the specific deoxygenation stimulus. We hypothesized that the magnitude of deoxygenation (Δ=nadir-baseline) would correlate with the magnitude of the reactive hyperemic response (Δ=peak-baseline), whereas the total deoxygenation (O2 debt, calculated as the total area under the curve of deoxygenation, below baseline) would correlate with the total reactive hyperemic response (area under the reactive hyperemia curve, above baseline).

In six (3M:3F) young healthy adults, we continuously measured forearm blood flow using doppler ultrasound on the brachial artery and muscle O2 saturation via near-infrared spectroscopy (MOXY) during three lengthening occlusion timed-reactive hyperemia tests (1, 5, or 10 minutes in duration). The magnitude of deoxygenation was significantly ($P<0.05$ via paired t-test) less during 1 min occlusion (-13±1.6%) compared to either 5 min occlusion (-67±14%; $P=0.0015$) or 10 min occlusion (-74±10%; $P=0.0004$), but 5 min vs 10 min occlusion were not different ($P=0.40$). Similarly, the magnitude of the RH was greater in both the 10 min occlusion (329±102 ml/min; $P=0.0004$) and 5 min occlusion (295±123 ml/min; $P=0.005$) vs 1 min occlusion (131±69 ml/min; $P<0.0001$), but were not different from one another ($P=0.11$). However, the total deoxygenation increased progressively from 1 min (-335±51 units), to 5 min (-10732±2209 units), to 10 min (-32357±5053 units; all $P<0.01$) as did the total reactive hyperemic response (1 min:1326±927 ml; 5 min:7865±4055 ml; 10 min:17447±9698 ml; $P<0.01$). These results suggest that two distinct deoxygenation factors, the absolute magnitude of and the total mismatch between O2 supply and demand, may guide human skeletal muscle's reactive hyperemic response profile. The increased duration in a deoxygenated state observed in the 10 min occlusion leads to a greater overall reactive hyperemia response, potentially mediated by increased muscle metabolite production and greater bioavailability of vasoactive products mediated by the fully deoxygenated erythrocytes.

Acknowledgements

I'd like to first like to thank my advisor Dr. Crecelius for her continuous support and guidance throughout my project as well as Dr. Beerse. In addition, I would like to thank all the participants who volunteered their time. Finally, I would like to thank the University of Dayton Honors Program and the College of Arts and Sciences Dean's Summer Fellowship for their support.

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Introduction

The human endothelium consists of a monolayer of cells that form the inner lining of all arteries, capillaries, and veins. Though it was once thought to be a simple barrier layer between the vasculature and circulating blood, it has now been shown to have a variety of endocrine-related functions. Notably, it has been shown to control blood fluidity, platelet aggregation, and vascular tone, as well as play a hand in immunology, inflammation, and angiogenesis (Félétou, 2011). Furthermore, in numerous studies that relate peripheral vasculature function with long-term clinical outcomes, endothelium dysfunction accurately predicts cardiovascular disease (CVD) and mortality (Anderson et al., 2011; Paine et al., 2016). Each of these studies used reactive hyperemia (RH), a well-studied method for assessing human endothelial function (Rosenberry et al., 2019). At a basic level, all RH tests consist of occluding arterial blood flow to a limb (usually an arm) and measuring the reperfusion rate of the skeletal muscle following a predetermined period of tissue ischemia (Rosenberry & Nelson, 2020). A quick rate of tissue reperfusion is indicative of optimal endothelial function. RH test can be conducted by various methods such as venous occlusion plethysmography, Doppler ultrasound (US) measurements of brachial artery velocity, peripheral artery tonometry, and NIRS measurements of muscle reperfusion (Rosenberry & Nelson, 2020). In all tests, a blunted reactive hyperemic response is indicative of endothelial dysfunction and subsequent cardiovascular disease (Anderson et al., 201; Paine et al., 2016).

Doppler ultrasound is one of the most common methods to measure reactive hyperemia in a clinical setting. Celermajer et al., in 1992, first presented their use of ultrasound to show reduced endothelial function, characterized by reduced vessel

dilation, in populations at a significantly increased risk for CVD (Celermajer et al., 1992). Since their initial findings, ultrasound technology has improved significantly. Now, with the use of B-mode imaging (the 2-d display of tissue and organ boundaries) and continuous blood velocity measurements, US is an invaluable tool for measuring RH (Hoskins et al., 2010, Rosenberry & Nelson, 2020). The two common methods for assessing the RH response include using US to measure arterial blood velocity post occlusion and calculating blood flow by taking continuous artery diameters in combination with arterial blood velocity. A variety of analysis methods have been used to quantify RH responses, including peak blood flow, peak blood velocity, velocity-time integrals, and average hyperemic velocity and flow following occlusion (Rosenberry & Nelson, 2020). Notably, many of these studies examine RH as a response to a predetermined time of occlusion (usually five minutes). While Doppler US is a well-studied tool for measuring RH, it requires a trained technician to operate and expensive equipment, making it a less accessible way of testing endothelial function.

A more recent technique for measuring RH is by using near-infrared spectroscopy (NIRS) devices. These devices measure the concentration and oxygenation status of light-absorbing chromophores, specifically myoglobin and hemoglobin (Barstow, 1985). UV light is emitted into the tissue, measuring its absorption spectra, which differ when heme is bound to oxygen and unbound (Barstow, 1985). This allows these devices to be placed noninvasively over a muscle and record the relative oxygen saturation of that tissue. The NIRS device used in this study (Moxy 3) is accurate on a 0-100% saturation scale making it appropriate for research studies (Feldmann et al., 2019). NIRS devices are used to assess the rate of skeletal muscle reperfusion, which has been shown to be a proxy for

blood flow (Bopp et al., 2014). Similar to the US, techniques to quantify RH vary; some studies have used reperfusion slopes (Mayeur et al., 2011), while others used the integral of reperfusion following the occlusive period (Rosenberry et al., 2018).

A novel study conducted by Roosenberry in 2018 found that the typical five-minute occlusive protocol used in clinical tests produced differing RH responses in younger versus older populations. However, when adjusted for total oxygen desaturation reached in the muscle, the two populations showed similar levels of RH. These results suggest that the RH response may be dependent on total vasodilatory stimulus, dictated by the extent of deoxygenation; which varies depending on the metabolic activity of the muscle (Rosenberry et al., 2018; Rosenberry et al., 2019; Mayeur et al., 2011). Furthermore, it has been shown that RH responses increase with increasing occlusion times (Leeson et al., 1997). Likewise, muscle oxygenation decreases linearly with time after occlusion of blood flow (Feldmann et al., 2019). The current clinical standard assesses the RH response using a standard 5 minute occlusion time. However, this may produce inaccurate results due to individual variations in metabolic activity producing different levels of muscle deoxygenation in a standard 5 minute occlusive period. A potentially more accurate test would assess the RH response to a standard level of muscle oxygen desaturation. Previous studies have made this secondary finding but further research is necessary to solidify the link between muscle deoxygenation and blood flow (Rosenberry et al., 2018). Therefore, the purpose of this study was to further validate the findings that total RH blood flow would increase with increasing occlusion times and muscle SmO2 (a proxy for total vasodilatory stimulus) would decrease. To test this

hypothesis we evoked varying magnitudes of deoxygenation through increasing arterial occlusion times of 1, 5, and 10 minutes and quantified the RH response to each stimulus.

Methods

Subjects

Six (3M:3F) young healthy subjects (**Table 1**) aged 20-22 years participated in this study. On the day of the lab visit, each participant was asked to fill out a health questionnaire to rule out any risks that would preclude them from participating. Additionally, each subject was explained the risks associated with the study and signed a waiver to participate. All subjects had abstained from food, caffeine, and alcohol in the twelve hours leading up to the lab visit. Following the initial health screening subjects had their weight and body composition measured (Tanita Corporation, 2014) and self reported their height.

Experimental Setup

During the course of the study, subjects laid supine with their left arm extended at heart level onto a table for blood flow data collection. NIRS devices, MOXY 3, (Fortiori Design LLC, 2015) were secured over the thickest portion of the subjects left and right forearms using black coban wrap to measure muscle oxygen saturation. The sensors on each device were aligned parallel with the forearm, per company guidelines. During the duration of the study we continuously measured heart rate with a three lead ECG, and blood pressure with finger plethysmography placed over the right middle finger and adjusted for height (Finapres Medical Systems, 2015). The subject had a rapidly inflating pressure cuff placed proximally over the upper left arm which was inflated to 200 mmHG

during periods of occlusion. Additionally, the subject had another pressure cuff wrapped around their left wrist inflated to 200mmHg during the duration of each trial to occlude all blood flow to the hand (D.E. Hokanson INC., 2010). During each trial, blood velocity was collected on the left brachial artery using a doppler ultrasound pencil probe (Compumedics Germany, 2018). Additionally, we estimated the diameter of the brachial artery using 3 caliper measurements made by a separate US probe in B-Mode imaging (GE Healthcare, 2014).

Protocol

All six participants underwent a series of occlusive periods of 1,5, and 10 minutes in a randomized order. Each trial began with a 15 minute rest period in order for their vital signs to stabilize. Following the 15 minute rest period, a blood pressure was taken using an automatic brachial cuff in order to calibrate the finger plethysmography pressure readings. At the start of each trial the wrist cuff was rapidly inflated to 200 mmHG and three minutes of resting measurements were recorded. Heart rate, beat-to-beat blood pressure, muscle oxygenation (SmO2%), and blood velocity were collected every second for the duration of each trial. At the end of the three minute rest period the upper arm cuff

was rapidly inflated to 200 mmHg, occluding all blood flow to the arm for a period of either 1, 5, or 10 minutes in a randomized order. At the end of each occlusive period the upper cuff was rapidly deflated and measurements continued to be taken for another 2.5 minutes in order to capture the reactive hyperemic response. In between successive trials, the wrist cuff was deflated and the participant allowed a 15 minute rest period before beginning the next trial.

Data and Statistical Analysis

To quantify the RH response we chose to look at the magnitude of the response given as the difference between peak blood flow post ischemia and resting blood flow (averaged over 30 sec before occlusion). We calculated blood flow (Eq. 1) using the equation for flow where v is the average blood velocity over a given second, and D is a standard brachial artery diameter measured via Doppler US at rest.

$$[\, v \times \pi \times \left(\frac{D}{2}\right)^2 \times 60s \quad] \qquad \text{Eq. 1}$$

Total blood flow was calculated using the integral of blood flow versus time over the two and half minutes after the ischemic period. The magnitude of deoxygenation was calculated as the difference between the minimum SmO2 value reached during occlusion and the resting value (the average resting muscle SmO2 during the thirty seconds prior to occlusion). Additionally, we quantified the total deoxygenation as the integral of the muscle SmO2 versus time during the occlusive period. To determine significance in our data we used a paired one tail t-test in each of our data sets on each of our dependent variables, (magnitude of deoxygenation, magnitude of RH, total deoxygenation, and total blood flow). The direction of the t-test was set in accordance with the expectation that

each dependent variable would increase with increasing occlusion times. Effect size was determined using Cohen's d.e. Significant differences were set at alpha=0.05.

Results

The magnitude of deoxygenation (Figure 1) was significantly less during 1 min occlusion (-13±1.6%) compared to either 5 min occlusion (-67±14%; t=10.067, d=4.11, P>0.001) or 10 min occlusion (-74±10%; t=15.462, d=6.312, P<.001), but 5 min vs 10 min occlusion were not different. Similarly, the magnitude of the RH (Figure 2) was greater in both the 10 min occlusion (329±102 ml/min; t=-8.559, d=3.494, P<0.001) and 5 min occlusion (295±123 ml/min; t=-4.770, d=1.947, P=.003) vs 1 min occlusion (131±69 ml/min), but were not different from one another (t=-1.913, d=0.781, P=0.057). However, the total deoxygenation (Figure 3) increased progressively from 1 min (-335±51 units), to 5 min (-10732±2209 units; t=11.686, d=4.771, P<.001) and from 5 min to 10 min (-32357±5053 units; t=15.421, d=6.394, P<.001). Likewise, the total reactive hyperemic response (Figure 4) increased from 1 min (1326±927 ml) to 5 min (7865±4055 ml; t=4.069, d=1.661, P=0.005) and from 5 min to 10 min (17447±9698 ml; t=3.830, d=1.563, P=0.006).

Discussion

This study found, as expected, that the magnitude of deoxygenation increased with increasing occlusion times in concurrence with previous studies (Barstow, 1985) (Rosenberry et al., 2019). When comparing the RH response to the magnitude of deoxygenation, we found a negative linear relationship between the two (Figure 5).

Around 5 minutes of occlusion, the magnitude of deoxygenation approached a minimum threshold indicated by the relatively small difference between the 5 and 10-minute trials. The SmO2 similarity between trials is likely due to the muscle metabolizing all available oxygen after 5 minutes. However, it is important to note that the occlusive period needed to reach a fully deoxygenated state is individual-specific, depending upon individual metabolic rates of skeletal muscle (Rosenberry et al., 2019). We found that even within a relatively similar population of young healthy adults one individual reached their physiological minimum SmO2 in 286s and another required 445s. We also demonstrated that the RH response increased with increasing occlusion times, which is in agreement with previous research (Leeson et al., 1997; Rosenberry et al., 2019). Interestingly, there was no significant difference between the 5 and 10-minute responses, indicating that the magnitude of RH may be dependent on the magnitude of deoxygenation.

Additionally, this study showed that the cumulative deoxygenation over time increased progressively with increasing occlusion times. One would expect that if RH was solely dependent on the magnitude of deoxygenation then the total RH response would show no significant difference between the 5 and 10-minute occlusive periods that reached similar SmO2 minimums. However, the total reactive hyperemia response showed significant increases between each increasing occlusive period. We found a negative linear relationship between the total RH and total cumulative deoxygenation (Figure 6). This suggests that the RH response may be dependent upon two factors; the magnitude of deoxygenation and the total cumulative deoxygenation. The mechanism for this result may lie in endothelial derived metabolites.

Many different physiological mechanisms are involved in the vasodilatory response seen during RH. A major contributor to this response is the production of endothelial-derived vasodilatory metabolites such as nitric oxide and vasodilatory prostaglandins (Engelke et al., 1996). During the occlusive phase of the test, tissue metabolism consumes oxygen lowering the SmO2; concurrently, these metabolites begin to build up and are partially responsible for the increase in vessel diameter. Previous studies have shown that maximal RH responses can be seen at just 4.5 minutes of occlusion, consistent with our findings that showed slight differences in RH magnitude after 5 minutes of occlusion (Leeson et al., 1997). We believe that the level of metabolite buildup present at 5 minutes is sufficient enough to induce maximal vasodilation which would account for the relatively small differences in RH magnitude past this point. During the ten-minute occlusion trial, the magnitude of RH is the same; however, the total RH response is greater. This is likely due to a further increase in vasoactive metabolites produced over the longer period of ischemia. The greater quantity of metabolites requires longer to clear the system and cease their local vasodilatory effects.

The current clinical standard for assessing endothelial function is a post-occlusion RH test (Roosenberry et al., 2018). However, the current method may be flawed due to individual differences in metabolism (Rosenberry et al., 2019, Rosenberry et al., 2017), making timed occlusion unreliable. This study further supports the findings that RH depends on both the magnitude of deoxygenation and the total deoxygenation. Further research is required to create an improved test using SmO2 as the stimulus rather than time. Such a test would demonstrate that given the same level of metabolic activity, an individual's endothelium produced fewer vasoactive products and, therefore, could be

diagnosed with endothelial dysfunction. The current test could misdiagnose individuals with relatively slow metabolisms as having endothelial dysfunction.

We acknowledge that the present study uses a relatively small sample size. However, we are confident that the results still bear significance due to the consistent findings with previous studies and the relatively large effect sizes. Another consideration of this study was using a single brachial artery diameter to approximate blood flow. While not ideal, all subjects had relatively similar brachial artery diameters, and we do not believe that the lack of continuous measurements affects our ability to make comparisons across individuals of the same treatment conditions. Future studies are needed to further verify these results using a larger population size and continuous diameter measurements.

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Figures

Table 1. Subject demographics including age, height, weight, BMI, and artery diameter.

Participant	Age	Height (m)	Weight (Kg)	BMI	Artery Diameter
S1	21.00	1.73	68.04	22.81	0.34
S2	22.00	1.75	81.60	26.55	0.40
S3	21.00	1.68	63.96	22.77	0.31
S4	22.00	1.68	72.57	25.84	0.33
S5	21.00	1.91	99.79	27.50	0.45
S6	20.00	1.63	74.39	28.14	0.30
Average	21.17	1.73	76.73	25.60	0.36
SD	0.75	0.10	12.78	2.32	0.06

Figure 1. Shows the magnitude of deoxygenation calculated as the minimum SmO2-resting SmO2.

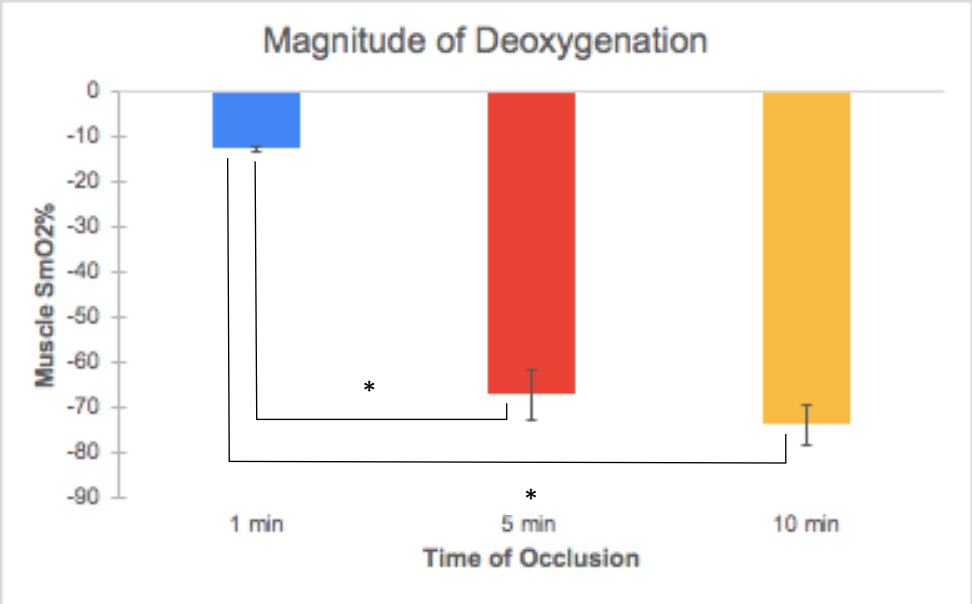

Figure 2. Magnitude of RH calculated as peak blood flow minus resting blood flow.

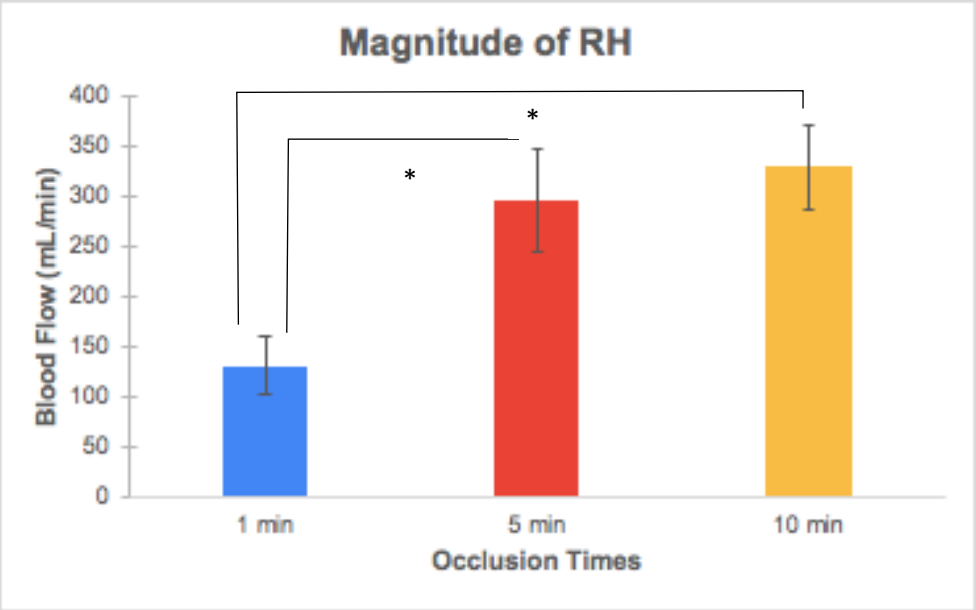

Figure 3. Total Deoxygenation given as the area under the curve of the SmO2.

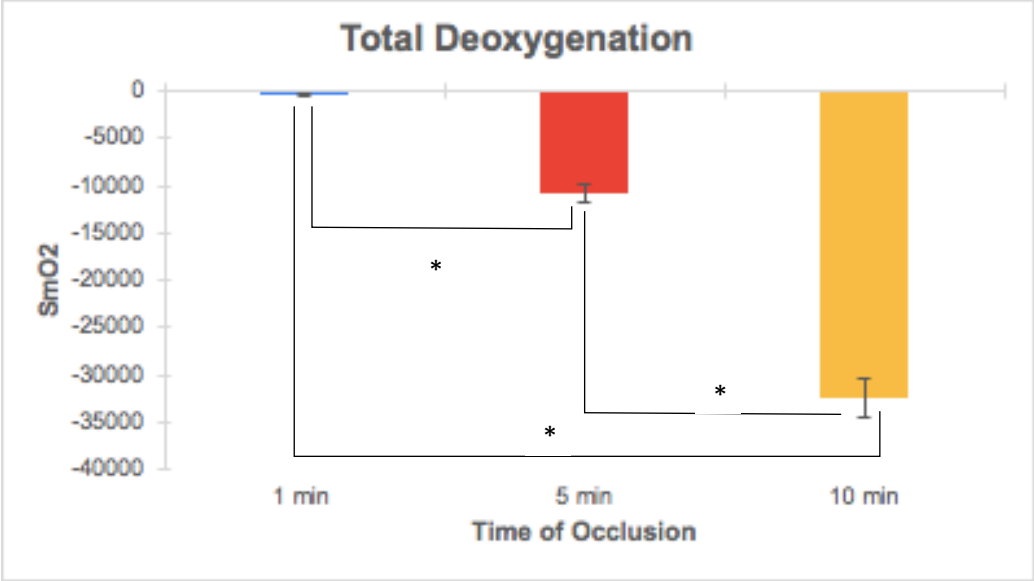

Figure 4. Total RH response calculated as the area under the curve of the blood flow 2.5 minutes after the ischemic period.

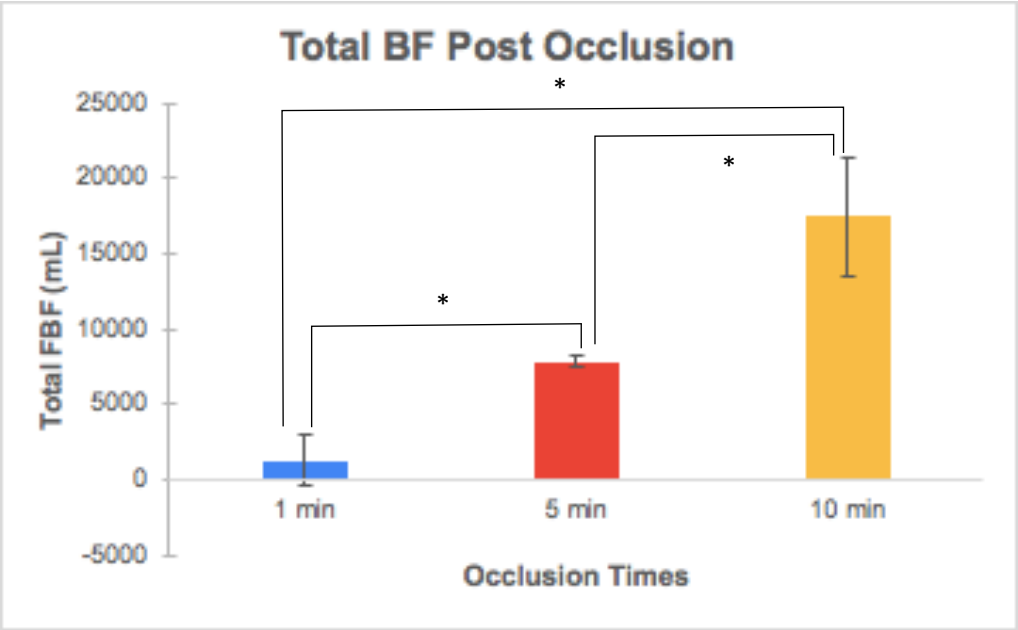

Figure 5. Demonstrates the negative linear relationship between magnitude of deoxygenation and the magnitude of reperfusion.

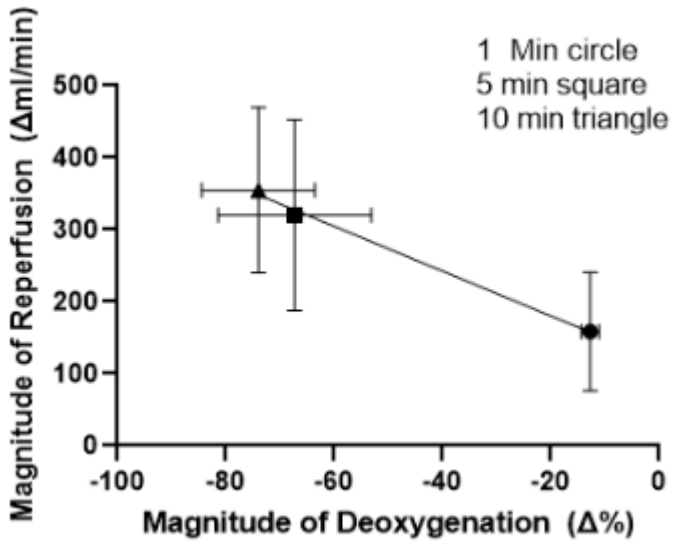

Figure 6. Demonstrates the negative linear relationship between the cumulative deoxygenation and the total RH.