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Permalink

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Journal

Microcirculation, 23(4)

ISSN

1073-9688

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Publication Date

2016-05-01

DOI

10.1111/micc.12277

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Peer reviewed



Published in final edited form as:

Microcirculation. 2016 May ; 23(4): 293–300. doi:10.1111/micc.12277.

Exercise and Repeated Testing Improves Accuracy of Laser Doppler Assessment of Microvascular Function Following Shortened (1-min) Blood Flow Occlusion

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Abstract

Objectives—To determine whether stability/accuracy of post-occlusive laser Doppler flowmetry following shortened, 1-min blood flow occlusion, increases in the post-exercise state or by averaging multiple measurements.

Methods—Six healthy adults (3F) underwent laser Doppler flowmetry 8 times at rest and following exercise, assessing post-occlusive (1-min occlusion) reactive hyperemia in the cutaneous microcirculation of the forefinger. Measured variables included: pre- and post-occlusion steady-state perfusion (Plat1, Plat2), maximum post-occlusive perfusion (Max), time to reach Max (PkT), and the ratio Max/Plat1.

Results—Stability/accuracy of all variables improved performing measurements after exercise ($P < 0.05$ Plat 1, Plat 2, Max and Max/Plat1). PkT and Max/Plat 1 displayed the greatest accuracy at rest ($26.6 \pm 5.1\%$ and $26.6 \pm 4.4\%$ average difference, %Diff, of single measurements from individual “true” means, respectively); for these variables, %Diff improved to 19.5 ± 5.3 and 17.6 ± 2.1 , respectively, following exercise. Overall, averaging multiple measurements performed at rest also improved stability/accuracy in all variables. This improvement was comparable to that obtained with a single measurement following exercise.

Conclusions—A standardized exercise stimulus prior to testing significantly improves stability/accuracy of laser Doppler flowmetry following shortened, 1-min blood flow occlusion. Our results suggest the possibilities of broader applications of exercise to optimize measurements from a variety of skin perfusion methodologies.

Keywords

Exercise; laser Doppler flowmetry; post-occlusive reactive hyperemia; reliability

INTRODUCTION

In clinical and research applications, the assessment of vascular function has become of major importance in diagnosing and following the evolution of a variety of pathologies. While acute or chronic changes in vascular function may occur in a variety of tissues

(cerebral cortex, cardiac and skeletal muscle, adipose tissue), their reliable and reproducible assessment often requires invasive or costly methodologies. Hence, relevant studies can often only be conducted indirectly on animal models (4). In humans, cutaneous microcirculation has been proposed as a surrogate marker of systemic microvascular function in disease states, such as diabetes (12), hypertension (17,18), and scleroderma (6,24). While controversy still exists as to whether the mechanisms modulating cutaneous vasodilation also closely reflect regulation of systemic endothelial function, particularly concerning the involvement of nitric oxide synthase (25,27), recent reports indicate how at least one NO synthase isoform (eNOS) mediates cutaneous NO-dependent vasodilation (5). An alternative explanation may also include activation of the endothelium-derived hyperpolarizing factor (EDHF) pathways, considered a possible backup vasodilator mechanisms to NO and prostaglandins (13).

Many non-invasive methods that can be used to investigate microcirculation of the skin exist. For example, capillaroscopy (7) and optical coherence tomography (8) can be used to visualize microvessels, and laser Doppler imaging (4), laser Doppler flowmetry (10), photoplethysmography (1), and near-infrared spectroscopy (26) can measure blood flow. Among them, laser Doppler flowmetry (LDF), has been gaining importance as a tool to assess endothelial function due to its simple administration, low cost per test, and minimal discomfort (10). In laser Doppler flowmetry, the tissue of interest is illuminated with a coherent laser light. Photons that interact with moving blood cells undergo Doppler shifts. Both Doppler-shifted and –unshifted photons are then diffusely scattered from the tissue, where a sensor interprets the resulting pattern as relative blood flow (16).

However, LDF has drawbacks which may affect reproducibility. Due to the fact that LDF evaluates perfusion in a small volume (20), and skin perfusion is spatially heterogeneous (23), variation on probe placement, particularly in skin areas of low capillary density, will introduce variability, and increase LDF sensitivity to movement. Different cutaneous districts have displayed differing inter-day variability in LDF measurements; forearm measurements, for instance, have displayed much greater variability and worse reproducibility as compared to measurements performed on the finger pad (19).

An additional issue particularly relevant to pediatric population is that LDF tests are commonly conducted in the post occlusive state, i.e. evaluating the reactive hyperemia that follows the transient occlusion of a vascular district. In this setting, stability of results is related to the duration of the blood flow occlusion (performed with a blood pressure cuff inflated at supra-systolic levels). Typical occlusion times of 3–5 minutes may cause considerable pain/discomfort, and therefore be particularly inadequate for children. Reducing the duration of the test to 1 min has been proposed as an alternative for pediatric studies (3), further increasing the need to optimize reproducibility.

Averaging the results of multiple tests, (making sure the probe is exactly placed in the same cutaneous area), could be an approach to reduce variability (14), but would of course imply the burden of additional time and cost. A different approach may be to perform the test in the presence of an alternative, standardized vasodilative stimulus, such as following an exercise challenge (9); this could substantially reduce the variability in resting

measurements, as well as eliminate the issue of variability from probe placement. To our knowledge, no study has attempted to systematically determine the contributions of exercise to the reproducibility of laser Doppler assessments of endothelial function, and compared this effect to the improvement in reproducibility obtainable via averaging multiple resting measurements in the same subjects.

The purpose of the present study was therefore to measure, in a group of healthy young subjects of both genders, a set of key variables of endothelial function using LDF during reactive post-occlusive hyperemia. As each participant repeated all measurements a minimum of eight times, both at rest and after a standard incremental maximal cycling exercise test, the study allowed to determine intra-individual variability of relevant variables, as well as the increase in stability obtainable in the post-exercise state and by averaging two or more consecutive measurements.

MATERIALS AND METHODS

Subjects

All studies were conducted at the University of California, Irvine Institute for Clinical and Translational Science (UCI ICTS). Study procedures were approved by the UC Irvine institutional review board, and informed verbal and written consents were obtained from all subjects prior to participation. Each of the 6 healthy subjects (3F, 23 \pm 3 yrs) participated in at least 8 baseline measurement studies and post-exercise studies, for a total of 51 visits. Prior to experimental sessions, subjects were asked to abstain from eating or consuming caffeinated beverages for 2h, and to patiently wait 15 min prior to starting measurements. All studies were conducted in a light- and temperature-controlled (22°C) room. For each subject, all studies were conducted > 24 h apart.

Measurements

Cutaneous perfusion was measured using an LDF system (Periflux 5000, Perimed, Järfälla, Sweden). A laser Doppler probe was adhered to the center of the proximal phalanx of the middle finger on the right hand. To minimize measurement variability due to the spatial heterogeneity of skin perfusion within each study visit, the location of the adhered probe during baseline measurements was marked for post-exercise measurements.

Vascular reactivity was measured with a post-occlusive hyperemia (PORH) test. All subjects were tested in the supine position; prior to perfusion measurements, a blood pressure cuff was placed at the right wrist. After the initial instrument setup and acclimatization period, baseline perfusion measurements were then taken for 5 min. The blood pressure cuff was then inflated at 220 mmHg for 1 minute, and then rapidly deflated. Post-occlusion perfusion measurements were then taken for at least 10 min. This set of procedures was performed twice, first in resting conditions, and then following a standard exercise challenge. Following exercise, perfusion measurements were timed so that the 1 min blood flow occlusion began exactly 8 min after exercise cessation. A typical response curve, along with a graphical representation of commonly measured parameters is shown in Figure 1.

Exercise consisted of a standard, incremental cycling challenge to exhaustion (Cycle Ergometer 800, SensorMedics). After a 2 min warm-up period of unloaded pedaling, resistance on the ergometer was increased every minute by one-tenth of individual pre-determined estimated maximum (based on standard predictive equations) (2), so that the incremental part of the tests lasted approximately 10 minutes. When subjects could not sustain a cycling speed of at least 60 revolutions/min, the incremental part of the challenge was considered concluded, and they were allowed a 2-min cool-down period of unloaded pedaling.

Data Analysis

We analyzed the following response PORH test response parameters, which we have defined in detail in Table 1: pre-occlusive plateau (Plat1), post-occlusive plateau (Plat2), peak perfusion (Max), time to peak perfusion (PkT), the ratio of Max/Plat1, and perfusion velocity (PVeI). For each variable, group averages were calculated including all individual measurement for each subject, and data from pre- and post-exercise states were compared via paired Student's T-tests. For repeated measurements, group averages were compared via a 2-way ANOVA, with repeated measures for the pre- and post-exercise states. If overall significance was found for either repeated measurements or exercise, comparisons were conducted via Student's T-tests with Bonferroni's correction for multiple comparisons.

Within each subject, for each analyzed metric the coefficient of variation (CV), as well as the average % difference of each measurement from the individual mean, was calculated separately in the pre- and post-exercise states. The CV for each metric was determined by dividing the standard deviation of an individual set of measurements by the mean of that same data set, then multiplied by 100 to convert the value into a percentage.

The increase in reproducibility obtainable via averaging results from multiple tests was determined via an *in silico* experiment, including a boot-strapping procedure utilizing Matlab software (The Mathworks, Inc, Natick, USA). First, for each variable, the mean of all measurements in each subjects was calculated. For instance, in our male subject XY, PkT was measured 9 times at rest, and the mean of the 9 measurements was 3.4 ± 0.4 sec. Each of the 9 individual measurements, of course, differed from the mean; the % differences of each measurement, as compared to the mean, were 3, 38, 9, 55, 15, 28, 27, 35 and 24%; on average, therefore, individual measurement differed from the mean all 9 measurements by 26%. 500 random samples of these 9 values were then drawn and averaged; then 500 random samples of the average of any two values in the series; and then 500 random samples of the average any 3 values in the series. The mean of each these three 500-sample sets was calculated, yielding, respectively, 26%, 18%, and 14%. This indicated that for this subjects, on average, result from a single study were likely to be within 26% of his/her true value; performing the study twice and averaging the results would reduce this discrepancy to 18%, and averaging three studies would further reduce it to 14%. A similar calculation could have been done simply comparing the average of the 9 single measurements to the averages of all possible two-value or three-value combinations in the series. This however would have generated three data sets with substantially different number of values (9 single measurements, 36 pairs and 84 triplets). Conversely, the boot-strapping procedure included

the same number of values in each set (500), which was sufficiently large to allow for convergence of the distribution of random samples.

RESULTS

As a group, participants displayed considerable homogeneity in the measured variables, both in the pre- and in the post-exercise states (Figure 2).

All three variables reflecting direct flow measurements (pre-, post-occlusion plateaus, and max perfusion), as expected, displayed a significant increase in the post exercise state. Conversely, the ratio of max perfusion to pre-occlusion plateau and the time to max perfusion were significantly reduced by exercise; perfusion velocity was not affected by exercise.

PkT and Max/Plat 1 were the variables with the lowest coefficients of variation (CV) in resting conditions (33 ± 6 and 36 ± 7 %, respectively), while other variables' CVs were progressively greater (Max, 47 ± 5 , PVel 55 ± 7 , Plat2 64 ± 13 , Plat 1 66 ± 14) (Figure 3). In all cases, CV's were quantitatively reduced during tests performed in the post-exercise state, although the reduction reached statistical significance only for Max.

Effect of exercise and study repetition of reproducibility of results

The average % difference of each measurement from the individual mean (%Diff) in resting conditions ranged from 26.6% (PkT and Max/Plat1) to 47.8% (Plat 1) (Figure 4).

Quantitatively, %Diff for all variables was markedly improved in the post exercise state (Figure 4), reaching statistical significance for Plat 1, Plat 2, Max/Plat1 and Max. PkT and Max/Plat1 were the only two variables in which %Diff in the post exercise state improved to values below 20% (to 19.5% and 17.6 % respectively; again, this improvement only was statistically significant for Max/Plat1, $p = 0.031$, pre vs post).

Averaging results from multiple studies also considerably reduced %Diff for all variables. In resting conditions, this reduction in %Diff became statistically significant for Plat1 and Plat 2 averaging results from two studies, and in PVel and Max averaging results from 3 studies. Again, the lowest absolute values were observed in PkT and Max/Plat1, in which averaging 2 studies performed in resting conditions resulted in %Diff values lower than 20% ($18.2\pm 3.7\%$ and $19.4\pm 3.8\%$, respectively; neither of these values, however, reflected a statistically significant improvement). Averaging results from multiple studies in the post exercise state resulted, intuitively, in markedly lower values of % Diff in all variables; while this improvement was statistically significant, with averages of 2 studies, only for Plat1 and Plat 2, the lowest absolute values were actually recorded in Max/Plat1 (12.2%), Max (15.1%) and PkT (15.6%).

From a more general point of view, in most instances, averaging two studies at rest resulted in %Diff values close to the values from a single post-exercise study, but never resulted in values under 20%.

DISCUSSION

This study aims to assess the use of exercise and repeated testing on the analysis of a PORH via LDF, using a shortened (1 min) blood flow occlusion time. The major findings of this study are (i) that stability of results can be improved in the post-exercise state or with repeated testing, and (ii) the gain in stability from exercise is similar to repeating the assessment twice.

We observed improvements in stability for all of our metrics. The largest reduction in CV following exercise occurred in Plat1, Plat2, and Max, the metrics displaying the greatest initial variability. A smaller but measurable decrease in the CV of PkT and Max/Plat1 was also observed. As these metrics were initially the most stable, the margin of improvement of their CV was understandably smaller. Taken as a whole, the general improvements from a standardized exercise stimulus may provide a foundation upon which a more accurate assessment of endothelial function could be conducted. Further, the larger improvements in the more unstable metrics we measured implies that exercise may allow for an analysis inclusive of additional variables (currently considered too unstable) generated from a PORH test. This would allow for a more comprehensive, and possibly more meaningful, assessment of endothelial function given a limited amount of perfusion data per subject.

Increasing the length of occlusion has been demonstrated to increase reproducibility of key metrics from PORH (11,21). For instance, Keymel et al. reported how maximal post-occlusive forearm perfusion was significantly increase prolonging occlusion from 1 to 3 min, with no further increase extending occlusion to 5 min (the latter, however, while not affecting amplitude, prolonged the duration of post occlusive dilation) (11).

In this study, therefore, performed in adults, with a 1 min occlusion, several considerations may be in order. First, as this protocol was used with a probe placed on the finger, while data may parallel systemic endothelial responses, the mechanisms in place may or may not be the same as those previously reported with forearm measurements (i.e. EDHF vs. NO mediated) (5,13,25,27). Further, our experimental stimulus resulted in a submaximal amplitude of post occlusive dilation; whether achieving maximal dilation is necessary for relevant endothelial function measurements, however, is not clearly established, and it is definitely possible that a well-characterized, submaximal 1-min post occlusive assessment may yield all necessary information. This context further underlines the possible importance of our post-exercise assessments: in this condition, the absolute increase in max post occlusive perfusion, as compared to rest, acts as a “functional extender” of the occlusion time, i.e. it generated the perfusion conditions that would be present at rest after a more prolonged occlusion time. In general, prolonging occlusion time has to be balanced with the need to prevent movement artifacts, which, if present, may result in the need for repeated testing. The pediatric population would be most relevant to this issue, where younger patients may have difficulty staying immobile during the numbing sensation that is associated with longer occlusion timings. Indeed, the limiting PORH occlusion to 60 sin children has been shown to minimize movement artifacts while still allowing meaningful assessments of endothelial function (3). Performing our study directly on children would have possibly further enhanced the strength of our results; the logistical complexity and discomfort of the required number of study

repeats in a vulnerable population, however, would have raised considerable regulatory issues. Further, performance of the study on healthy young adults facilitates comparison with available published data, most of which is in adults, while maintaining applicability to younger populations.

Several prior studies in this area have reported, in addition to simple perfusion flux, cutaneous vascular conductance (CVC), which is defined as perfusion flux divided by the mean arterial pressure at the time of the study. The use of CVC has been previously demonstrated to account for wide variations in blood pressure, resulting in more stable inter-subject results (15,19). We acknowledge that our results would have been strengthened by the addition of this variable in our study. Our subjects are active young adults in a university setting, with homogenous, demonstrated, stable vascular health. We therefore posit that the relative improvement in data quality would not have been as strong as in other, more variable populations. Exercise, however, which was used here as a tool to stabilize data reproducibility, is indeed a stimulus that physiologically increases blood pressure, and we therefore acknowledge the potential usefulness of CVC in this setting. We therefore definitely endorse its use in any future studies, particularly including challenging or vulnerable populations.

Despite the fact that our study procedures were conducted in a carefully controlled experimental setting, the instability of our measured metrics remained relatively high, both in pre- and post-exercise conditions. This would imply that garnering meaningful results would be even more difficult in a standard clinical setting, where attention to patients is often unfortunately limited, and the performance of multiple tests sessions, or the addition of an exercise protocol, may add an unrealistic burden in terms of time and cost. However, the general improvements from using a standardized exercise format show great potential in a research setting, where the exercise stimulus could be used in addition to other methods of limiting extraneous variability in skin perfusion measurements. Further, this study posits that the idea that using exercise as a tool in reducing baseline variability may extend to other methodologies that look at endothelial function, or at other local or systemic indices of vascular function. Further opportunities of more generalized applications also stem from the fact that the exercise protocol would not necessarily require expensive, specialized ergometers, as used in this study; but could be standardized using much more basic, minimal equipment (e.g. stepping blocks). These alternative approaches could still generate a ~ 10-min testing format effectively reproducing an incremental, maximal, exercise challenge. Maximizing the vasodilative effect of exercise per se is important, we feel, in the context of a shortened occlusive period. It should also be mentioned that the indirect assessment of endothelial function has been shown to benefit, in terms of reproducibility, from the use of a local heater stabilizing skin temperature during testing (22). While this methodology was not used in the present study, it is likely that its implementation would further enhance several of the reported effects.

In conclusion, our study was aimed at maximizing the reproducibility of the LDF assessment of PORH during a shortened occlusion protocol (1 min of forearm blood flow occlusion, tailored toward pediatric populations). Our results show that providing a standardized exercise stimulus prior to testing significantly improves the stability of

endothelial function measurements. The simplicity of implementing exercise and meaningful improvement of results prompts the need for additional inquiry related to exercise and other skin perfusion methodologies.

Perspectives

The non-invasive measurement of endothelial function via LDF is an important tool in the study of cardiovascular health. Inconsistent data reproducibility across subjects, as well as discomfort associated with prolonged blood flow occlusion during testing, have often limited applicability of this methodology, especially in pediatric populations. We report how performing the test after a standardized exercise protocol markedly improves reproducibility and stability of LDF results (the improvement being comparable to that obtained by averaging two separate tests performing in resting conditions).

Acknowledgments

Grant Numbers: National Institutes of Health P01HD048721, K24DK085223, TL1TR001415, UL1TR001414

The authors thank all UCI ICTS nursing staff, laboratory personnel, and specialists (Barbara Bodenhofer, A. Diane Capobianco, Connie Parido, Georgia Bachman, Milagros Ibarrolaza, Bridgette Duarte, and Frank Zaldivar) for their support.

LIST OF ABBREVIATIONS

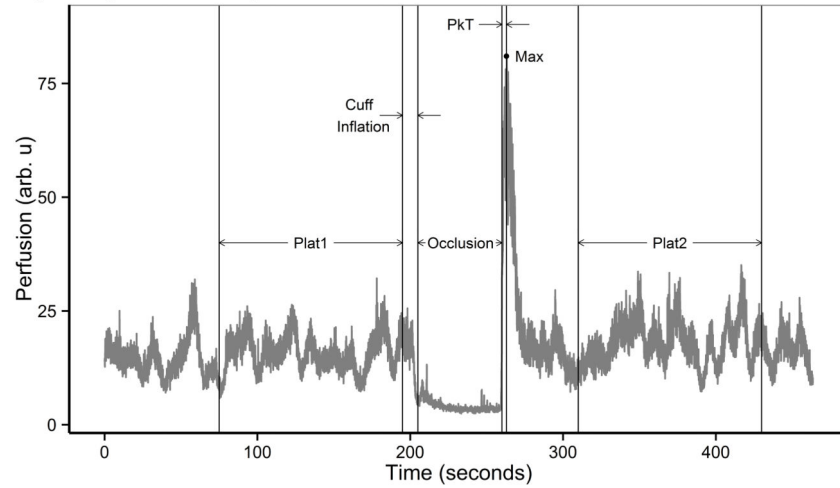
EDHF	endothelium-derived hyperpolarizing factor
LDF	laser Doppler flowmetry
PORH	post occlusive reactive hyperemia
CV	coefficient of variation
%Diff	average percent difference from the individual mean (for either pre- or post-exercise states)
Plat1	pre-occlusive plateau
Plat2	post-occlusive plateau
Max	peak perfusion
Max/Plat1	ratio of peak perfusion to pre-occlusive plateau
PkT	time to peak perfusion
PVel	perfusion velocity
CVC	cutaneous vascular conductance

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A) Sample LDF Output**FIGURE 1.**

A representative blood flow perfusion curve during a post-occlusive hyperemia test. Key time periods are noted with arrows. Plat1, Plat2: steady-state flow values before and after occlusion, respectively; Max: peak values of post-occlusive perfusion; PkT: time to reach peak perfusion after end of occlusion.

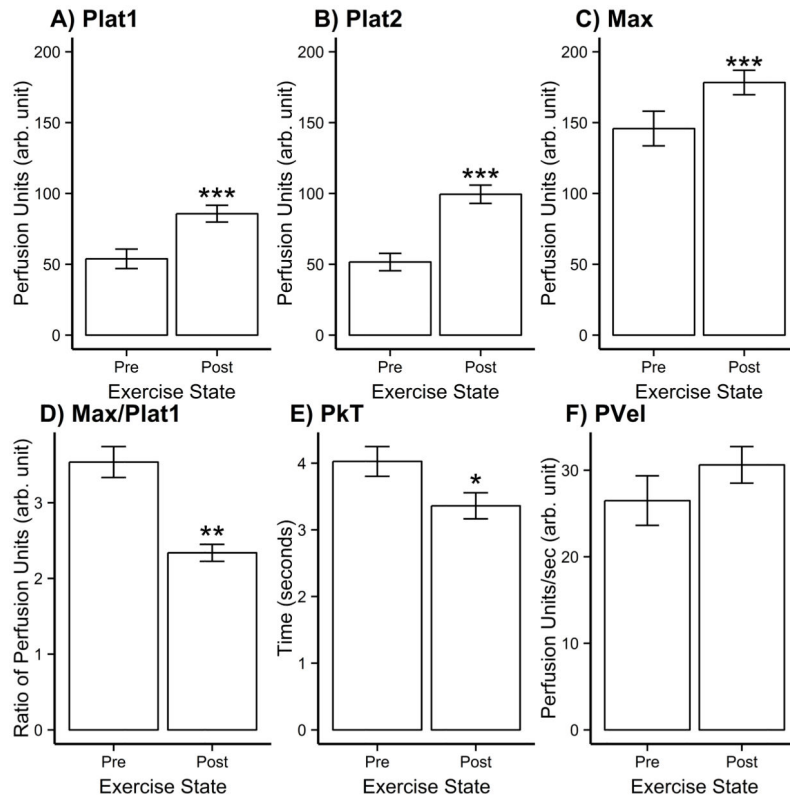


FIGURE 2.

Perfusion variables in 6 healthy subjects before and after a standardized ramped exercise test. Data are overall group means \pm SE of 51 resting and 51 post-exercise measurements (a minimum of 8 resting and 8 post-exercise tests per subject). Plat1, Plat2: steady-state flow values before and after occlusion, respectively; Max: peak values of post-occlusive perfusion; PKT: time to reach peak perfusion after end of occlusion; PVel, ratio of post-occlusive increase in flow over Plat1 and PKT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

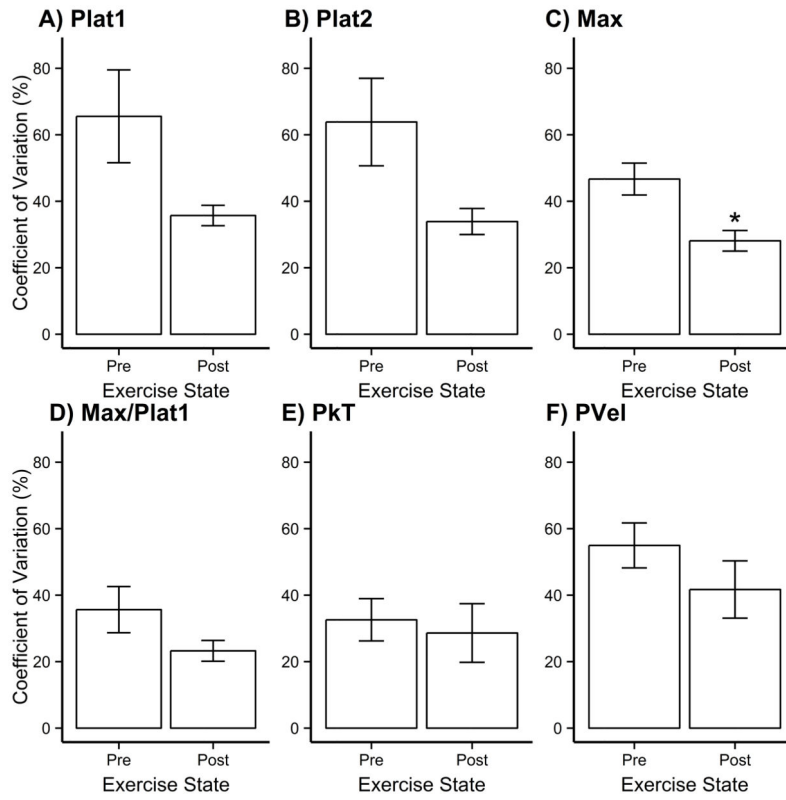


FIGURE 3. Coefficients of variation of perfusion variables before and after a standardized ramp test. Data are overall group means \pm SE of 51 resting and 51 post-exercise measurements (a minimum of 8 resting and 8 post-exercise tests per subject). Plat1, Plat2: steady-state flow values before and after occlusion, respectively; Max: peak values of post-occlusive perfusion; PkT: time to reach peak perfusion after end of occlusion; PVel, ratio of post-occlusive increase in flow over Plat1 and PkT. * $p < 0.05$.

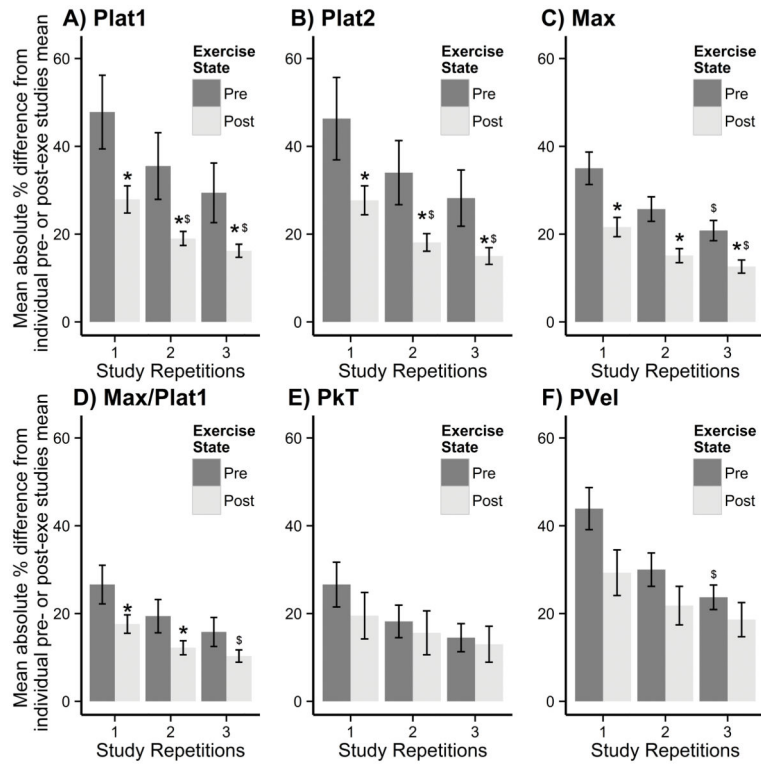


FIGURE 4. Mean percentage differences (%Diff) of LDF variables using either single measurements, or the average of 2 or 3 measurements (study repetitions), in pre and post-exercise conditions. For each subject, %Diff is the average discrepancy between any single measurement (or average of any 2 or three measurements) and that subject’s “true value (i.e. the mean of 8 or 9 measurements). Data are group means \pm SE. *, P<0.05, post- vs pre-exercise state; \$, P<0.05 vs one study repetition. Specific p-values are listed in Table 2.

Measured laser Doppler flowmetry response variables and how we defined them for this project.

TABLE 1

Variable	Definition
Pre-occlusive plateau (Plat1)	The mean value of perfusion prior to the induced occlusion in the current PORH test.
Post-occlusive plateau (Plat2)	The mean value of perfusion following the induced occlusion and the decay to a steady-state in the current PORH test. The time point of the decay was determined by loosely fitting a third degree polynomial to the post-occlusive data ($r = .25$), obtaining the second derivative of the curve, and selecting the inflection point of the first concavity.
Ratio of peak perfusion over pre-occlusive plateau (Max/Plat1)	The value of the current pre-occlusive plateau divided by the post-occlusive plateau.
Peak perfusion (Max)	The maximal value of perfusion in the current PORH test.
Time to peak (PKT)	The time from the end of the induced occlusion to the maximal value of perfusion in the current PORH test.
Perfusion Velocity (PVel)	The value of the absolute difference of the peak perfusion and pre-occlusive plateau divided by the time to peak.

TABLE 2

P-values following analysis of %Diff across exercise state and study repetitions.

Variable	Comparison	Pre-Exercise			Post-Exercise		
		1 Study	2 Studies	3 Studies	1 Study	2 Studies	3 Studies
Plat1	pre-exe vs. post exe				0.01*	0.03*	0.04*
	vs. 1 study repetition		0.15	0.06		0.02*	0.003**
	vs. 2 study repetitions		0.28				0.10
Plat2	pre-exe vs. post exe				0.05	0.04*	0.04*
	vs. 1 study repetition		0.16	0.07		0.02*	0.004**
	vs. 2 study repetitions		0.28				0.14
Max	pre-exe vs. post exe				0.01*	0.004**	0.01*
	vs. 1 study repetition		0.04*	0.004**		0.02*	0.003**
	vs. 2 study repetitions		0.10				0.13
Max/Plat1	pre-exe vs. post exe				0.03*	0.04*	0.05
	vs. 1 study repetition		0.12	0.04*		0.04*	0.01*
	vs. 2 study repetitions		0.24				0.19
PKT	pre-exe vs. post exe				0.24	0.37	0.41
	vs. 1 study repetition		0.11	0.04*		0.30	0.18
	vs. 2 study repetitions		0.23				0.35
Pvel	pre-exe vs. post exe				0.10	0.18	0.24
	vs. 1 study repetition		0.02*	0.002**		0.15	0.06*
	vs. 2 study repetitions		0.11				0.30

* p < 0.05,

** p < 0.01