

## Variability of flow-mediated dilation measurements with repetitive reactive hyperemia

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**Abstract:** To capture the response of an acute intervention, multiple post intervention measurements of flow-mediated dilation (FMD) must be performed. The effect of repetitive reactive hyperemia on endothelial function and the measurement of FMD are unknown. The purpose of this investigation was (1) to examine the effect of repetitive reactive hyperemia on brachial artery FMD and (2) to determine whether brachial artery FMD is stable during a 2-h morning period. We investigated FMD in 20 apparently healthy college students on three randomized treatment days every 30 min ( $T_{30}$ ), 60 min ( $T_{60}$ ), and 120 min ( $T_{120}$ ) throughout a 2-h morning period (08.00 h to 10.00 h). An ANOVA ( $p > 0.05$ ) and ICC ( $> 0.40$ ) were both needed to confirm no difference among repetitive reactive hyperemia treatments. In response to repetitive reactive hyperemia, there was no difference ( $p = 0.307$ ; ICC  $> 0.40$ ) within the first and last FMD measurements of each treatment condition or between treatment conditions ( $p = 0.344$ ; ICC  $> 0.40$ ). FMD was similar ( $p = 0.348$ ) throughout the 2-h morning period. In conclusion, repetitive reactive hyperemia over a 2-h period has no effect on FMD measurements in apparently healthy college students. In addition, this study found no time trends for FMD measurements during the 2-h morning period to allow for pre/post intervention FMD measurements.

**Key words:** blood flow; endothelium; ischemia; repetitive reactive hyperemia

### Introduction

Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilators, in particular nitric oxide (NO)<sup>1</sup> and is seen in various clinical populations such as hypertension,<sup>2,3</sup> diabetes,<sup>4</sup> coronary artery disease,<sup>5,6</sup> and obesity.<sup>7</sup> The measurement of flow-mediated dilation (FMD) via ultrasound has been established as a reliable non-invasive measurement of endothelial function<sup>8</sup> and has been shown to correlate with invasive testing of coronary artery endothelial function.<sup>9,10</sup>

Numerous interventions that improve cardiovascular risk factors and reduce cardiovascular morbidity and mortality have been shown to increase brachial artery reactivity measured by FMD, thus improving endothelial function.<sup>11</sup> Repeated measures of FMD

are often required for the clinical study of various acute interventions on endothelial function, such as diet or exercise. When multiple measurements are to be performed over a 1-day period, several confounding variables may exist and need to be controlled.

Reported by the Guidelines for the Ultrasound Assessment of Endothelial-Dependent Flow-Mediated Vasodilation of the Brachial Artery<sup>12</sup> to decrease sensitivity and establish more reliable FMD measurements, room temperature, previous physical activity, medications, vitamin supplementation, smoking, and food intake should be controlled. The guidelines mention nothing regarding a diurnal variation of FMD. Conversely, there is inconsistent evidence to suggest a morning variation of brachial artery reactivity within the time period measured.

To capture the response of an acute intervention, multiple post intervention measurements of flow-mediated dilation (FMD) must be performed. There are limited data investigating the variability of FMD measured several times within any given period of the day. Manipulating the hyperemic flow patterns through multiple repetitive reactive hyperemia procedures may have an effect on the sensitivity of the vasodilator response of the endothelium. Thus, the purpose of this study was (1) to investigate the effect of repetitive reactive hyperemia on brachial artery FMD and (2) to

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determine whether brachial artery FMD is stable during a 2-h morning period. It was hypothesized that (1) repetitive reactive hyperemia has little or no effect on brachial artery FMD, and (2) there is no variation of brachial artery FMD during a 2-h morning period between 08.00h and 10.00h, both of which were investigated in apparently healthy college students.

## Methods

### Experimental design

Figure 1 illustrates the study design. All subjects reported to the Indiana University Clinical Exercise Physiology Laboratory at 07.45 h for a 2-h period of FMD testing on each day of the three randomized treatment conditions:  $T_{30}$  (testing every 30 min),  $T_{60}$  (testing every 60 min), and  $T_{120}$  (testing every 120 min); five, three, and two measurements, respectively. Repetitive reactive hyperemia ( $n = 16$ ) analysis compared the first (A) and last (B) FMD measurements for each treatment condition. Morning variation analysis ( $n = 20$ ) compared all five FMD measurements (08.00, 08.30, 09.00, 09.30, 10.00h) obtained from condition  $T_{30}$ . To control for confounding variables, prior to testing, subjects were instructed to (1) fast for 12 h; (2) abstain from exercise, caffeine, and tobacco for 12 h; and (3) awake between 06.00 and 07.00 h.

### Subjects

Sixteen apparently healthy college students participated in the repetitive reactive hyperemia phase of the study, and four additional apparently healthy college students participated in the morning variation phase of the study ( $n = 20$ ). Demographics of these subjects are presented in Table 1. Subjects were excluded if they were taking vasoactive medication<sup>12</sup> and/or their brachial artery diameter was  $>5.0$ .<sup>12</sup> Written informed consent was obtained from each subject prior to participation in the study. All procedures were

	Time				
	0800	0830	0900	0930	1000
$T_{30}$	x	x	x	x	x
$T_{60}$	x		x		x
$T_{120}$	x				x
	A		B		

**Figure 1** Experimental protocol for repetitive reactive hyperemia and morning variation. ( $T_{30}$  indicates FMD measurements every 30 min;  $T_{60}$  indicates FMD measurements every 60 min;  $T_{120}$  indicates FMD measurements every 120 min; (x) represents FMD measurements; (A) indicates the first FMD measurement; (B) indicates the last FMD measurement.)

**Table 1** Subject demographics and brachial artery characteristics for repetitive hyperemia and morning variation protocols.

	Repetitive hyperemia $n = 16$	Morning variation $n = 20$	$p$ -value
Sex, male/female	9 / 7	10 / 10	NS
Age, years	22.5 $\pm$ 0.8	23.1 $\pm$ 0.73	NS
Height, cm	174.3 $\pm$ 2.3	173.5 $\pm$ 2.0	NS
Weight, kg	71.3 $\pm$ 3.4	71.8 $\pm$ 3.0	NS
Body mass index, $\text{kg m}^{-2}$	23.3 $\pm$ 0.6	23.8 $\pm$ 0.7	NS
Systolic blood pressure, mmHg	109.2 $\pm$ 2.7	109.8 $\pm$ 2.6	NS
Diastolic blood pressure, mmHg	70.5 $\pm$ 1.7	72.1 $\pm$ 1.6	NS
Baseline brachial artery diameter, mm	3.72 $\pm$ 0.11	3.57 $\pm$ 0.09	NS
Flow-mediated dilation, %	9.56 $\pm$ 0.81	10.65 $\pm$ 0.82	NS

Values are mean  $\pm$  SEM.  
NS, not significant.

approved by the Indiana University Committee for the Protection of Human Subjects prior to study commencement.

### Flow-mediated dilation preparation

Subjects were instructed to lie supine in a dark, climate-controlled room (22–24°C), with their right arm extended out laterally. Two adult-size sphygmomanometric blood pressure cuffs were placed on the subject's (1) left upper arm for blood pressure measurements and (2) right forearm to induce reactive hyperemia. Each subject underwent an acclimation phase ( $<10$  min) where blood pressures were taken in the left arm every 2 min. The steady state of brachial artery reactivity was assumed when systolic blood pressures were within 5 mmHg on two consecutive readings.

### Brachial artery images

The brachial artery was imaged longitudinally, 2–10 cm above the antecubital fossa by a 2D high resolution Sonoace Pico (Universal Medical Systems, Bedford Hills, NY, USA) ultrasound system, using a 7 MHz linear transducer. Once a clear artery image was obtained, three still images were captured on the ultrasound prior to occlusion (baseline). Another three still images were captured at 45 s, 60 s, and 90 s (12 images total).

### Reactive hyperemia

The blood pressure cuff placed on the subject's right forearm was inflated 50 mmHg above their systolic blood pressure. After 5 min of occlusion, the pressure was rapidly released to allow for reactive hyperemia to occur. The brachial artery was continuously imaged

throughout the whole procedure. Three still images were captured for each post hyperemic time period at 45 s, 60 s, and 90 s (nine images total).

### Brachial artery analysis

For each still image, five brachial artery diameters were measured in evenly spaced segments, approximately every 0.25 cm using B-mode as previously described.<sup>13,14</sup> The average of all the measurements for each time period (baseline, 45 s, 60 s, and 90 s) were calculated and recorded for that individual time period. FMD was expressed as the per cent change in diameter from baseline using the highest post hyperemic time period diameter value.

### Reproducibility of FMD measurements

Reproducibility was previously obtained from 60 independent images captured via ultrasound using five subjects. Brachial artery still images were measured by two independent observers. Inter-observer reliability yielded an Intra-Class Correlation Coefficient (ICC) of 0.9768 with a variation of 2.32%. Intra-observer reliability yielded an ICC of 0.978 and 0.973, and a variation of 2.22% and 2.7% for the two independent observers, respectively.

### Reproducibility of reactive hyperemic flow

Hyperemic blood flow (ml/min) was analyzed at 15 s following the onset of reactive hyperemia using a subset ( $n = 6$ ) of the same population studied throughout the present investigation. Blood flow was calculated by multiplying the cross-sectional area of the vessel ( $\pi d^2/4$ ) by the hyperemic velocity (cm/s) of the Doppler flow signal and the constant 60. The Doppler flow signal was corrected for an isonation angle of 70°.

### Statistical analysis

Repetitive reactive hyperemia comparisons were made using a two-way (treatment  $\times$  time) repetitive measures analysis of variance (ANOVA) (SPSS Inc., Chicago, IL, USA: v. 11.5). Mauchly's test of sphericity was used to identify homogeneity of variance among the sample population. One-way repeated measures ANOVA was performed for hyperemic blood flow measurements on the subset of six subjects. Intra-class correlations were performed on repetitive first (A) and last (B) measurements of each individual treatment condition as well as all treatment conditions combined. ICC values of  $<0.40$ ,  $0.4-0.75$ , and  $>0.75$  represent poor, fair-to-good, and excellent agreements, respectively.<sup>15</sup> An ANOVA ( $p > 0.05$ ) and fair-to-good ICC ( $0.40-0.75$ ) were both needed to confirm no difference among first and last measurements within each treatment. FMD measurements collected during condition  $T_{30}$  were analyzed using a one-way repeated measure ANOVA. All data are expressed as mean  $\pm$  SEM. Statistical significance for all data was set at  $p < 0.05$ .

### Results

FMD homogeneity of variance was assumed (Mauchly's  $W = 0.418$ ) for all 20 apparently healthy college students analyzed, 16 of whom participated in the three treatment days for repetitive hyperemia. Subject's demographic and brachial artery characteristic data are displayed in Table 1. No differences ( $p > 0.05$ ) were seen among gender subgroups and no difference existed ( $p = 0.249$ ,  $F_{1,5} = 1.7$ ) among hyperemic flow stimulus among the investigated subsample. Baseline and peak hyperemic flows for morning time periods are presented in Table 2.

**Table 2** Baseline and peak hyperemic blood flow differences within subjects throughout the 2-h morning time period.

Doppler flows	Time (h)					p-value
	08.00	08.30	09.00	09.30	10.00	
Mean baseline (ml/min)	71.6 $\pm$ 22.8	73.7 $\pm$ 16.0	76.3 $\pm$ 15.5	83.4 $\pm$ 15.8	76.7 $\pm$ 17.0	NS
Subject 1	-41.1	-35.4	-41.7	-51.4	-45.9	
Subject 2	-2.9	12.8	29.8	38.6	35.6	
Subject 3	-43.2	-12.0	-17.8	-23.8	-35.9	
Subject 4	108.4	59.3	48.5	50.3	60.9	
Subject 5	-11.3	21.4	20.3	2.5	2.1	
Subject 6	-9.9	-46.0	-39.1	-16.2	-16.7	
Mean post occlusion (ml/min)	719.2 $\pm$ 81.7	672.4 $\pm$ 50.9	740.1 $\pm$ 79.4	707.0 $\pm$ 91.9	795.1 $\pm$ 104.9	NS
Subject 1	19.0	45.2	99.9	231.5	244.0	
Subject 2	-324.7	-352.2	-429.1	-472.0	-475.2	
Subject 3	1878.7	134.6	564.5	795.1	1523.3	
Subject 4	-861.0	-598.9	-447.4	-366.8	-583.6	
Subject 5	-352.4	-319.3	-375.3	-256.6	-351.2	
Subject 6	-359.6	1090.7	587.4	68.9	-357.3	

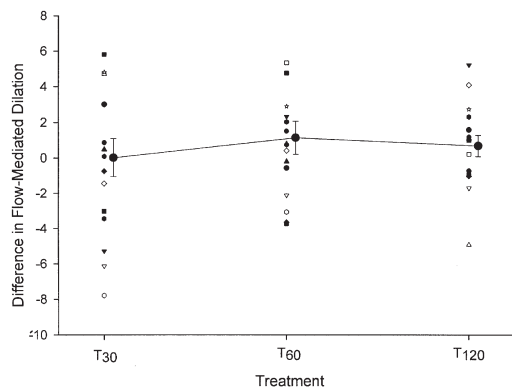
Values are mean  $\pm$  SEM.

Subject data are individual differences from the mean.

NS, not significant.

### Repetitive reactive hyperemia

Figure 2 displays the mean and each individual FMD difference between 08.00h and 10.00h for each treatment condition. The baseline brachial artery diameter, absolute change in brachial artery diameter, and FMD corresponding to the first (A) and last (B) FMD measurements within each treatment condition are presented in Table 3. There was no difference ( $p = 0.307$ ,  $F_{1,15} = 1.17$ ) observed between measurements A and B



**Figure 2** Mean ( $\pm$ SEM) and individual FMD difference between the first (08.00h) and last (10.00h) measurement for each treatment condition. (T<sub>30</sub> indicates FMD measurements every 30 min; T<sub>60</sub> indicates FMD measurements every 60 min; T<sub>120</sub> indicates FMD measurements every 120 min.)

**Table 3** Brachial artery diameters and flow-mediated dilation for the first (A) and last (B) measurements of each treatment condition.

	A	B	<i>p</i> -value
T <sub>30</sub>			
Baseline diameter, mm	3.69 $\pm$ 0.12	3.64 $\pm$ 0.13	NS
Absolute change, mm	0.35 $\pm$ 0.04	0.33 $\pm$ 0.03	NS
Flow-mediated dilation, %	9.59 $\pm$ 1.00	9.58 $\pm$ 0.98	NS
T <sub>60</sub>			
Baseline diameter, mm	3.71 $\pm$ 0.12	3.69 $\pm$ 0.12	NS
Absolute change, mm	0.34 $\pm$ 0.02	0.29 $\pm$ 0.03	NS
Flow-mediated dilation, %	9.19 $\pm$ 0.67	8.07 $\pm$ 0.88	NS
T <sub>120</sub>			
Baseline diameter, mm	3.77 $\pm$ 0.11	3.71 $\pm$ 0.12	NS
Absolute change, mm	0.37 $\pm$ 0.02	0.33 $\pm$ 0.03	NS
Flow-mediated dilation, %	9.88 $\pm$ 0.76	9.18 $\pm$ 0.76	NS

Values are mean  $\pm$  SEM.

NS, not significant.

(A) Indicates first measurement within treatment condition.

(B) Indicates last measurement within treatment condition.

and no difference ( $p = 0.344$ ,  $F_{1,15} = 0.956$ ) observed between each treatment condition. The ICCs demonstrated significant within-subject variability, which is presented in Table 4. There was a weak effect for the treatment conditions ( $\omega^2 = -0.001$ ), time ( $\omega^2 = 0.004$ ), and interaction ( $\omega^2 = -0.011$ ), displaying a low power of 0.200, 0.168, and 0.122 for each variable source, respectively.

### Morning variation

Twenty subjects were analyzed for their morning variation. Morning baseline brachial artery diameter, absolute change in brachial artery diameter, and FMD are reported in Table 5. Subjects exhibited a stable ( $p = 0.348$ ,  $F_{1,19} = 0.924$ ) FMD response throughout the 2-h morning period. There was a very weak effect ( $\omega^2 = -0.003$ ) among the five morning periods measured within T<sub>30</sub> also displaying a low power of 0.280.

### Discussion

The primary purpose of this investigation was (1) to investigate the effect of repetitive reactive hyperemia on brachial artery FMD and (2) to determine whether brachial artery FMD is stable during a 2-h morning period. The main findings of the present investigation based on analysis criteria are (1) repetitive reactive hyperemia, as a stimulus of brachial artery reactivity, has little or no effect on FMD measurements taken every 30 min, every 60 min, or every 120 min throughout a 2-h morning period, and (2) there is no significant morning variation of brachial artery FMD during the 2-h period between 08.00h and 10.00h. These findings may provide additional support in developing the acute intervention paradigm.

The strength of our findings can be illustrated by comparing the variation of FMD among multiple days to the existing literature. The daily variation in baseline FMD (08.00h) for the three treatments ranged from 4.4% to 7.5%, which corresponds to the acceptable range reported by Sorensen et al.<sup>16</sup> The finding that repetitive reactive hyperemia has no effect on FMD measurements allows for consecutive post

**Table 4** Variability of intra-class correlation coefficients among each treatment day and all treatment days combined.

	ICC
T <sub>30</sub>	0.587
T <sub>60</sub>	0.450
T <sub>120</sub>	0.811
All combined	0.624

ICC, intra-class correlation coefficients; T<sub>30</sub>, FMD testing every 30 min; T<sub>60</sub>, FMD testing every 60 min; T<sub>120</sub>, FMD testing every 120 min.

**Table 5** Variability of brachial artery diameters and flow-mediated dilation for a 2-h morning period.

Morning variation	Time					p-value
	08.00	08.30	09.00	09.30	10.00	
Baseline, mm	3.69 ± 0.12	3.62 ± 0.11	3.67 ± 0.11	3.66 ± 0.11	3.63 ± 0.12	NS
Absolute change, mm	0.34 ± 0.03	0.35 ± 0.03	0.32 ± 0.03	0.31 ± 0.03	0.32 ± 0.03	NS
Flow-mediated dilation, %	9.25 ± 0.86	9.71 ± 0.81	8.83 ± 0.72	8.39 ± 0.78	9.01 ± 0.90	NS

Values are mean ± SEM.  
NS, not significant.

intervention FMD measurements without affecting the brachial artery reactivity outcome. The finding that there is a stable FMD response throughout the 2-h morning period where pre and post intervention FMD measurements can be taken is of equal importance.

There has been conflicting evidence to support the idea of diurnal variation of endothelial reactivity as a confounding factor associated with FMD.<sup>17,18</sup>

Otto et al<sup>17</sup> found a significant increase in FMD from 06.00 h to 11.00 h, whereas Etsuda et al<sup>18</sup> found no difference in FMD between 08.00 h and 12.00 h. Nonetheless, a limitation of these studies is that prolonged periods of time ( $\geq 4$  h) were allowed to pass between FMD measurements. The 2-h morning period between 08.00 h and 10.00 h suggested by the present study is free of confounding morning variation of endothelial function. This stable time period can assist when investigating the time course associated with brachial artery endothelial reactivity post acute intervention.

The development of FMD as a non-invasive measurement of brachial artery reactivity has increased the ability to identify and detect cardiovascular disease before the symptoms arise.<sup>19</sup> FMD is a very sensitive measurement as differences are noted between various cohorts based on technical aspects of the FMD measurement.<sup>20</sup>

The smaller baseline artery diameter seen in women, leading to an increased and possible false-negative FMD, has also questioned the reliability and sensitivity of this measurement; although, it is accepted that FMD varies among subjects. It is important to note that there were no differences throughout the present investigation seen among gender subgroups. In addition, the importance of controlling for confounding variables is imperative to adequately perform the FMD measurement. In the present study, subjects were instructed to fast for 12 h, abstain from exercise, caffeine, and tobacco for 12 h, and awake between 06.00 and 07.00 h, all prior to testing. By controlling for as many variables as possible, it allowed us to examine potential confounding variables that have been overlooked in the past.

### Study limitations

The findings from the present investigation may not be generalized to other laboratories or other

investigational settings assuming the population investigated. The data are based on young healthy adults who appear to have similar FMD responses to older healthy adults. The inability to ECG gait and capture simultaneous Doppler blood flows with our ultrasound system at this time may present another limitation, although the inter-observer reproducibility and the baseline variability of FMD are consistent with the literature standards. In addition, the sub-sample ( $n = 6$ ) investigated solely on Doppler analysis suggests that our occlusion procedure generates a consistent hyperemic flow stimulus throughout the 2-h morning period. The present investigation used the minimal suggested sample size. The guidelines<sup>12</sup> report that population samples between 20 and 30 subjects are typically needed in a crossover design to see significant differences in FMD. The present study used the minimum number of subjects required, yet data suggest a combination of small effect size, low power, and insignificant F-ratio, which confirms the strength of the results; whereas, a large effect size with a small sample size finding no statistical significance would have been of concern. Nonetheless, the non-existence of such potential confounding variables that have been assumed throughout the past research has warranted the need for the present methodological investigation.

### Conclusion

In conclusion, these results, independent of sample size, suggest that performing repetitive reactive hyperemia every 30, 60 or 120 mins over a 2-h period has no significant effect on FMD measurement. In addition, this study found no time trends for FMD measurements using these time intervals during the 2-h morning period in which the response of an acute intervention can be investigated. Future investigators may want to extend this 2-h morning period to the 6-h period used in the diet intervention studies.<sup>21,22</sup> This methodological study may contribute to the acute paradigm protocols of investigating the absolute effects of FMD under various conditions, as well as identifying the time course associated with brachial artery reactivity, both in response to an acute intervention.

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