

RESEARCH ARTICLE

Fluctuation in shear rate, with unaltered mean shear rate, improves brachial artery flow-mediated dilation in healthy, young men

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Holder SM, Dawson EA, Brislane Á, Hisdal J, Green DJ, Thijssen DH. Fluctuation in shear rate, with unaltered mean shear rate, improves brachial artery flow-mediated dilation in healthy, young men. *J Appl Physiol* 126: 1687–1693, 2019. First published May 2, 2019; doi:10.1152/jappphysiol.00009.2019.—Increase in mean shear stress represents an important and potent hemodynamic stimulus to improve conduit artery endothelial function in humans. No previous study has examined whether fluctuations in shear rate patterns, without altering mean shear stress, impacts conduit artery endothelial function. This study examined the hypothesis that 30-min exposure to fluctuations in shear rate patterns, in the presence of unaltered mean shear rate, improves brachial artery flow-mediated dilation. Fifteen healthy men (27.3 ± 5.0 yr) completed the study. Bilateral brachial artery flow-mediated dilation was assessed before and after unilateral exposure to 30 min of intermittent negative pressure (10 s, -40 mmHg; 7 s, 0 mmHg) to induce fluctuation in shear rate, while the contralateral arm was exposed to a resting period. Negative pressure significantly increased shear rate, followed by a decrease in shear rate upon pressure release (both $P < 0.001$). Across the 30-min intervention, mean shear rate was not different compared with baseline ($P = 0.458$). A linear mixed model revealed a significant effect of time observed for flow-mediated dilation ($P = 0.029$), with exploratory post hoc analysis showing an increase in the intervention arm (Δ FMD $+2.0\%$, $P = 0.008$), but not in the contralateral control arm (Δ FMD $+0.5\%$, $P = 0.664$). However, there was no effect for arm ($P = 0.619$) or interaction effect ($P = 0.096$). In conclusion, we found that fluctuations in shear patterns, with unaltered mean shear, improves brachial artery flow-mediated dilation. These novel data suggest that fluctuations in shear pattern, even in the absence of altered mean shear, represent a stimulus to acute change in endothelial function in healthy individuals.

NEW & NOTEWORTHY Intermittent negative pressure applied to the forearm induced significant fluctuations in antegrade and retrograde shear rate, while mean shear was preserved relative to baseline. Our exploratory study revealed that brachial artery flow-mediated dilation was significantly improved following 30-min exposure to intermittent negative pressure. Fluctuations in blood flow or shear rate, with unaltered mean shear, may have important implications for

vascular health; however, further research is required to identify the underlying mechanisms and potential long-term health benefits.

endothelial function; flow-mediated dilation; fluctuations; shear rate

INTRODUCTION

Hemodynamic stimuli play an important role in inducing functional and structural changes in the arterial wall via endothelial cell signal transduction (12). More specifically, increased mean shear stress represents a key stimulus for vascular adaptation, for example in response to exercise training (4, 12, 35). Manipulating shear rate through exercise or heating has provided *in vivo* evidence that elevation in mean shear rate mediates acute (13, 34) and chronic (19) improvement in endothelial function, measured by flow-mediated dilation (FMD). In addition to levels of mean shear stress, the pattern of shear stress is important, since increasing the antegrade shear component was associated with improved FMD, while increasing retrograde and oscillatory shear is associated with impaired FMD (22, 31).

Recently, Sundby and colleagues (27) showed that exposure to intermittent negative pressure [10-s negative pressure (-40 mmHg), 7-s atmospheric pressure] causes fluctuations in patterns of blood flow and shear rate. More specifically, increased antegrade and mean blood flow (velocity) was present at the onset of negative pressure, followed by marked reduction in antegrade and mean blood flow (and increase in retrograde blood flow) upon release of the negative pressure. Interestingly, frequent use of intermittent negative pressure in patients with lower-limb ischemia and ulcers is associated with improved wound healing (25, 26, 28). These clinical effects suggest that fluctuations in blood flow and shear stress patterns may impact vascular health in humans. Unfortunately, these studies did not control for potential increases in mean shear levels. Therefore, it remains unclear whether these observations are linked to repetitive exposure to fluctuations in shear, or whether observations were simply explained through increases in mean shear stress levels.

To the best of our knowledge, no previous study in animals or humans has directly examined whether fluctuations in blood

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flow and shear stress patterns, in the presence of unaltered mean blood flow and shear rate, impacts upon endothelial function. Therefore, we assessed the effect of 30-min exposure to intermittent negative pressure, which mediates fluctuations in blood flow and shear rate patterns through the brachial artery, on FMD [a measure of largely nitric oxide-mediated, endothelial function (11)] in healthy young men. We hypothesized that fluctuations in blood flow and shear stress patterns would induce improvement in brachial artery endothelial function. Since fluctuations in mean shear stress are relevant to many activities of daily living, we planned this study to provide insight into the potential clinical relevance of fluctuations in shear stress as a hemodynamic stimulus for improvement in vascular health in vivo.

MATERIALS AND METHODS

Participants. Fifteen healthy men (age 27.3 ± 5.0 yr) were recruited for the study. All participants were nonsmokers, not taking medication and/or supplements known to influence the cardiovascular system, and free from cardiovascular/metabolic disease risk factors. Based on a prescreening health questionnaire, participants were excluded if they had poor circulation (including diagnosis of peripheral vascular disease or Reynaud's disease). Each participant provided written informed consent before taking part in the experimental procedure. The research study was ethically approved by the Liverpool John Moores School of Sport and Exercise Science Research Ethics Committee and adhered to the Declaration of Helsinki.

Experimental design. After 15 min of supine resting, we bilaterally examined brachial artery endothelial function using the FMD test (29). This was followed by a 10-min rest period to allow blood flow and diameter to return to baseline levels. Subsequently, following a 1-min recording of baseline diameter and blood flow velocity, subjects underwent a 30-min intervention involving intermittent negative pressure (i.e., left arm), while the right arm served as a control arm. Within 2 min of this intervention, we repeated bilateral brachial artery FMD testing.

Preparations. Prior to the laboratory visit, all participants were instructed to refrain from strenuous exercise for at least 24 h, alcohol for 12 h, avoid all caffeinated products for 8 h and food products high in polyphenols for 24 h. Participants reported to the quiet, temperature-controlled laboratory after fasting for at least 6 h. After reporting to our laboratory, stature and body mass were recorded to the nearest 0.1 unit using a stadiometer and digital scales, respectively. Body mass index (BMI) was calculated as body mass in kilograms divided by stature in meters squared (kg/m^2).

Brachial artery flow-mediated dilation. Brachial artery FMD was measured in accordance with contemporary expert-consensus guidelines (29). Following 15 min of supine rest, left and right brachial artery diameter was assessed simultaneously via high-resolution duplex ultrasound (Terason u-smart 3300, Teratech, Burlington, MA) with a 10- to 12-MHz linear array probe. B-mode images were obtained and optimized, and the probe was held in the same position for the duration of the test. After 1 min of baseline measurement, occlusion cuffs, connected to a rapid inflator (Hokanson, Bellevue, WA), placed around both forearms, distal to the humeral epicondyle, were inflated to a pressure of 220 mmHg for 5 min. Recording was resumed 30 s before cuff deflation, and FMD was recorded for a further 3 min post cuff deflation. All measurements were taken by the same experienced operators within participants. Bilateral FMD was repeated following the 30-min intervention period.

Brachial artery diameter and shear rate. High-resolution ultrasound (Terason u-smart 3300; Teratech) was used to examine brachial artery diameter and shear rate as described above. Following the preintervention FMD, the participant's skin was marked to ensure a consistent ultrasound probe position and therefore artery segment during the

visit. Furthermore, the ultrasound machine settings remained constant (i.e., depth and Doppler cursor position) to assume the same probe angle while imaging. Bilateral artery diameter and shear rate were recorded for 1-min baseline, and repeated at 5-min intervals during the 30-min intervention period.

Intervention. During the laboratory visit, participants rested in the supine position with both arms extended away from their body to $\sim 80^\circ$, with their palms facing upward for optimal ultrasound imaging of the brachial artery. During the 10-min rest period following the preintervention FMD, the left arm was placed inside a rigid plastic cylinder (8.5×40 cm) connected to a pressure control box (FlowOx, Otivio AS, Oslo, Norway; Fig. 1). The cylinder was sealed around the forearm with a thermoplastic elastomer (TPS-SEBS). The arm was exposed to repeated bouts of negative pressure (-40 mmHg; 10 s negative pressure, 7 s atmospheric pressure) for 30 min (~ 105 full cycles of negative pressure).

Blood pressure. Blood pressure and heart rate were recorded continuously during the protocol from the right (control) arm index/middle finger using a Portapres (Finapres Medical Systems BV, Amsterdam, The Netherlands). This data were displayed, recorded, and exported using PowerLab software (ADInstruments, Australia). The difference in blood pressure and heart rate was calculated from a 1-min recording before the intervention period started, and the last minute of the intervention.

Data analysis. All FMD data analysis was performed blinded by the same observer, using a specialized custom-designed edge-detection and wall-tracking software, of which the reproducibility and validity have been demonstrated elsewhere (39). This software tracks the vessel walls and blood flow velocity trace in B-mode frames via pixel density and frequency distribution algorithm. An optimal region of interest to be analyzed was selected by the sonographer, chosen on the basis of the quality of the image, in regards to clear distinction between the artery walls and lumen. The FMD was defined as the maximum percentage change in artery diameter from baseline to peak during the 3 min post cuff release. The software automatically calculated the relative diameter change, time to peak (following cuff release) and shear rate area under the curve (SRAUC). Despite the initial region of interest selection being operator determined, the remaining analysis was independent of operator bias.

Brachial artery diameter and shear rate were analyzed using the custom-designed software described above. The region of interest location (selected by the operator) remained consistent for each 1-min recording within participants. Using markers placed by the operator, the software calculated the average artery diameter and shear rate across the minute recordings. The fluctuations in shear stress were



Fig. 1. Photo of the experimental set-up. The participant lay supine with both arms extended for optimal ultrasound scanning of the brachial artery. Ultrasound machines and probes remained consistent throughout the study (Terason usmart 3300, Teratech, Burlington, MA) with 10- to 12-Hz probes. Furthermore, the settings on the ultrasound machine (i.e., depth, Doppler cursor position) were maintained for the duration of the laboratory visit. The participant's left arm was inside the rigid cylinder, connected to a pressure control box (not seen in the image) and exposed to 30 min of intermittent negative pressure, while the right arm served as a control.

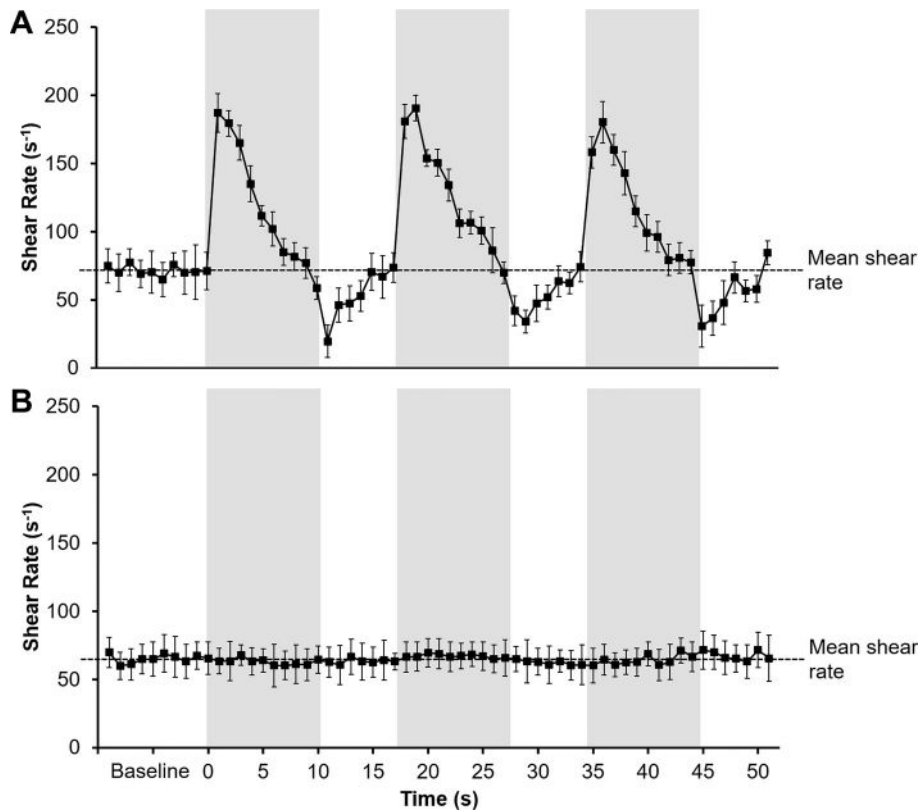


Fig. 2. Shear rate data of the brachial artery in the intervention arm (A) and control arm (B), calculated as 1-s averages at rest, followed by 3 cycles of intermittent negative pressure (gray bars: negative pressure) in 15 healthy young men. Values are means \pm SE. In A, note the clear fluctuations in brachial artery shear rate, with higher levels of mean and antegrade shear rate during (the first part of) negative pressure, followed by a rapid decline and normalization of mean and antegrade shear rate upon release of the pressure. B demonstrates no change in shear rate in the control arm during the intermittent negative pressure intervention. Mean shear rate is presented as the dashed line.

analyzed as an average during the application of negative pressure (10 s; On), atmospheric pressure (7 s; Off), and the full cycle, then repeated for the 3 full cycles captured during each 1-min recording. These processes were repeated for each time point during the intervention. Mean (\pm SE) shear rate data at baseline and during 3 cycles of intermittent negative pressure are presented in Fig. 2.

Statistical analysis. Statistical analysis was conducted using IBM SPSS version 25 (SPSS, Chicago, IL). Allometric scaling was performed on FMD data to control for differences in baseline diameter (2, 3). A linear mixed model with covariate control for SRAUC and scaled baseline diameter determined the main effect for time (Pre-Post) and arm. A general linear model assessed the changes in blood pressure and heart rate across the intervention period. Paired *t*-tests determined the difference in antegrade and retrograde shear during intermittent negative pressure compared with baseline in both arms. Statistical significance was recognized when a *P* value < 0.05 was observed. Data are presented as means \pm SE unless stated otherwise.

RESULTS

Subject characteristics are presented in Table 1.

Table 1. *Subject characteristics of the participants*

Parameter	Value
Age, yr	27.3 \pm 5.0
Height, m	1.75 \pm 0.06
Body mass, kg	75.1 \pm 7.5
BMI, kg/m ²	24.4 \pm 2.0
Systolic blood pressure, mmHg	115 \pm 3
Diastolic blood pressure, mmHg	62 \pm 7
Mean arterial pressure, mmHg	80 \pm 5
Heart rate, beats/min	52 \pm 8

Values are means \pm SD; *n* = 15 participants. BMI, body mass index.

Brachial artery blood flow and shear rate. There were no significant changes across the 30-min intervention in heart rate (means \pm SD: 52 \pm 7 vs. 54 \pm 8 beats/min, *P* = 0.47) or in systolic (129 \pm 9 vs. 135 \pm 12 mmHg, *P* = 0.16), diastolic (55 \pm 8 vs. 59 \pm 9 mmHg, *P* = 0.36) or mean blood pressure (80 \pm 8 vs. 84 \pm 9 mmHg, *P* = 0.23). Negative pressure was associated with a significant increase in mean shear rate, while pressure release was followed by a significant decrease in mean shear rate, to levels below baseline (“pressure on”: $\Delta + 34.2$ s⁻¹, “pressure off”: $\Delta - 26.5$ s⁻¹; both *P* < 0.001; Fig. 3A). Consequently, mean shear rate across the intervention period was not different from baseline (“pressure on/off cycle”: $\Delta + 3.8$ s⁻¹; *P* = 0.458). In the control arm, negative pressure did not change mean shear from baseline levels (“pressure on”: $\Delta + 1.6$, *P* = 0.805; “pressure off”: $\Delta + 3.5$ s⁻¹, *P* = 0.613). Therefore, mean shear rate remained unchanged throughout the intervention period compared with baseline (“pressure on/off cycle”: $\Delta + 2.5$ s⁻¹, *P* = 0.702; Fig. 3B).

When examining shear patterns, negative pressure increased antegrade shear rate (*P* < 0.001) and decreased retrograde shear rate (*P* = 0.006; Fig. 3A). Upon pressure release, compared with baseline levels, a decrease in antegrade shear rate and increase in retrograde shear rate was found (*P* = 0.003 and *P* < 0.001, respectively). As a result, mean antegrade and retrograde shear rate across the 30-min intervention period was not different from baseline (*P* = 0.504 and 0.777, respectively). Antegrade and retrograde shear rate remained unaltered from baseline in the control arm during “pressure on” (antegrade: $\Delta + 2.5$ s⁻¹, *P* = 0.730; retrograde: $\Delta - 1.9$ s⁻¹, *P* = 0.190) and “pressure off” (antegrade: $\Delta + 1.9$ s⁻¹, *P* = 0.779; retrograde: $\Delta - 2.0$ s⁻¹, *P* = 0.164; Fig. 3B). Therefore, mean

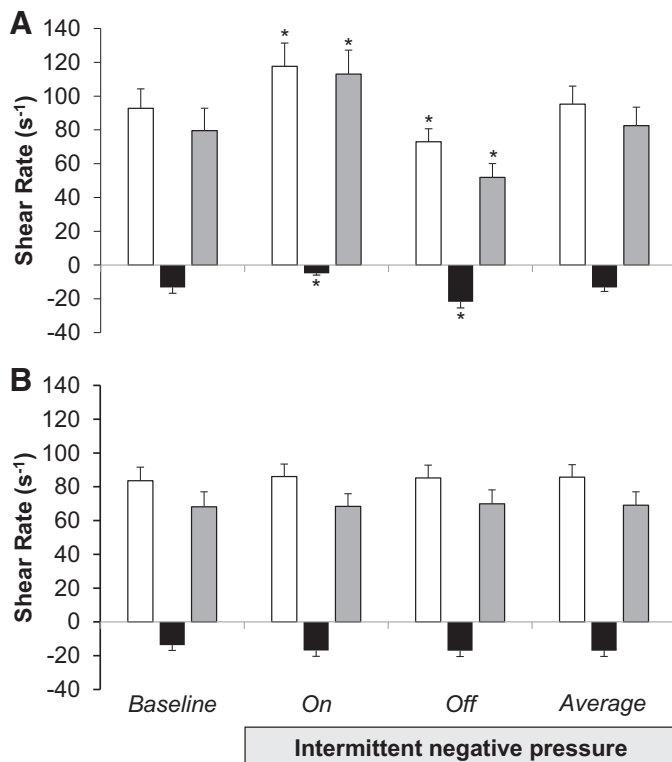


Fig. 3. Presentation of average levels of antegrade (white bars), retrograde (black bars), and mean (gray bars) shear rate at baseline and during the intermittent negative pressure intervention in the intervention arm (A) and control arm (B) of 15 healthy young men. Data during the intermittent negative pressure were presented during negative pressure ("on"), during pressure release ("off") and as the average across the entire 30-min intervention ("average"). Error bars represent SE. Paired *t*-tests determined differences in shear rate compared with baseline. *Significantly different from baseline at $P < 0.05$.

antegrade and retrograde shear rate was not different from baseline across the intervention (antegrade: $\Delta +2.2 \text{ s}^{-1}$, $P = 0.750$; retrograde: $\Delta -1.9 \text{ s}^{-1}$, $P = 0.173$).

Brachial artery FMD. Linear mixed model analysis revealed a significant main effect for time ($P = 0.029$; F -ratio = 5.146), while no effect was observed for arm ($P = 0.619$; F -ratio = 0.251) or interaction effect ($P = 0.096$; F -ratio = 2.906). Post hoc exploratory analysis revealed a significant increase in FMD in the intervention arm ($\Delta +2.0\%$, $P = 0.008$), while no change was observed in the control arm ($\Delta +0.5\%$, $P = 0.664$). Individual FMD responses are presented in Fig. 4, and all associated parameters (mean and 95% confidence intervals) are presented in Table 2.

DISCUSSION

We show that application of intermittent negative pressure to the forearm increases antegrade blood flow and shear rate, while pressure release mediates increased retrograde blood flow and shear rate measured at the brachial artery, relative to baseline and the contralateral control arm. Despite these marked fluctuations in blood flow and shear rate patterns throughout the 30-min intervention, mean blood flow and shear rate were not different from baseline. We therefore successfully preserved average resting levels of flow and shear rate, despite inducing fluctuations of these variables. Although ex-

ploratory in nature, we observed improved brachial artery FMD as a result of these fluctuations in blood flow and shear rate, an effect that was not apparent in the contralateral control limb. Taken together, these findings suggest that fluctuations in shear rate, independent of mean blood flow and shear rate, may impact acute vascular function in healthy young individuals. While further research is required, this contributes to improving our understanding of shear stress as an important hemodynamic stimulus in the adaptation of vascular health in humans *in vivo*.

Our findings regarding the impact of cyclical negative pressure are in line with a previous study in the lower limbs (27). Importantly, our study adds the novel knowledge that these fluctuations were associated with improvements in endothelial function, as measured with the brachial artery FMD. Blood pressure and heart rate remained unaltered during the intervention period, effectively excluding the possibility that systemic factors contributed to our observations. To further support this notion, no changes in brachial artery blood flow or shear rate were found in the contralateral arm. This strongly suggests that the mechanisms contributing to the increase in FMD in the intervention arm relate to local effects (i.e., fluctuations in shear rate) rather than systemic/circulating factors.

Our novel results may be somewhat surprising, in that the fluctuations in shear rate were not accompanied by changes in mean shear rate, but still caused an increase in FMD. In our previous work, supported by studies in animals (21, 38), we consistently found that changes in mean shear rate are essential to change FMD (31, 34). More specifically, selective increases in antegrade shear rate (and therefore mean shear rate) were related to improved FMD (13, 34), while an isolated increase in retrograde shear rate (i.e., lower mean shear rate) was associated with a dose-dependent decrease in brachial and femoral artery FMD (22, 31). One potential explanation for the increase in FMD is the relative larger importance of increases in antegrade shear rate compared with changes in retrograde shear rate. To support this idea, moderate-intensity cycling exercise acutely increases retrograde shear rate (10, 30), followed by normalization after ~15 min with a concomitant

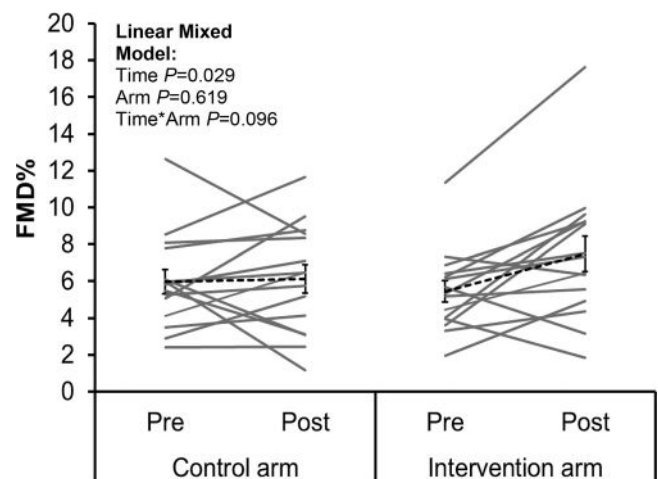


Fig. 4. Individual brachial artery flow-mediated dilation (FMD) responses to 30-min intermittent negative pressure in the intervention and control arms of healthy young individuals ($n = 15$). Black dashed line represents mean change in FMD. Error bars represent SE. A linear mixed model determined the main effect for time and arm.

Table 2. Brachial artery FMD for the intervention and control arm before and after 30-min exposure to unilateral intermittent negative pressure in healthy young individuals

	Intervention Arm		Control Arm		P Values		Time × Arm
	Pre	Post	Pre	Post	Time	Arm	
Baseline diameter, mm	4.04 (3.82–4.26)	4.02 (3.79–4.24)	3.82 (3.60–4.05)	3.79 (3.57–4.01)	0.671	0.002	0.957
Peak diameter, mm	4.26 (4.03–4.48)	4.31 (4.09–4.54)	4.07 (3.84–4.30)	4.05 (3.82–4.27)	0.797	0.001	0.603
FMD, %	5.5 (3.9–7.0)	7.5 (5.9–9.0)	6.4 (4.9–8.0)	6.9 (5.4–8.5)	0.029	0.619	0.096
SRAUC, s ⁻¹ × 10 ³	19.3 (15.0–23.5)	17.9 (13.6–22.1)	17.1 (12.8–21.3)	17.5 (13.2–21.7)	0.762	0.428	0.572
Time to peak, s	48 (40–56)	43 (35–51)	43 (35–51)	47 (39–55)	0.950	0.919	0.217

Data are presented as mean (95% confidence intervals); $n = 15$ individuals. P values refer to a linear mixed model to examine the main effect of Time (pre-versus postintervention), Arm (intervention arm versus contralateral control arm), and the interaction effect between time and arm (Time × Arm). FMD, flow-mediated dilation; SRAUC, shear rate area under the curve.

increase in antegrade shear rate (23). Nonetheless, acute or chronic performance of cycling exercise (i.e., 30-/40-min bouts) leads to improvement in brachial artery FMD (4, 12). This evolving hypothesis that changes in antegrade shear rate may be relatively more important than changes in retrograde shear rate warrants further investigation.

Another explanation for our findings relates to the importance of fluctuations in shear rate patterns, rather than mean shear rate. In the microcirculation, previous work used mathematical simulation to support the concept that fluctuations of capillary blood flow, rather than steady-state conditions, improve oxygenation of tissue (36). Follow-up work in humans examining skin perfusion and oxygenation demonstrated that periodic fluctuations in vasomotion may be beneficial for local oxygenation (32). In conduit arteries, some studies have found that enhanced external counterpulsation increased shear rate fluctuations and FMD in the brachial artery (5, 15). However, these changes were also accompanied by an overall increase in mean shear rate, making it impossible to isolate the impact of fluctuations per se (i.e., in the absence of changes in mean shear). Finally, indirect support for a potential clinically relevant, beneficial effect on vascular health for these fluctuations is provided by the observation of improved wound healing upon repeated exposure to intermittent negative pressure (26, 28). These observations may contribute to improved microcirculatory blood flow and therefore the delivery of oxygen and nutrients to promote wound healing (25, 26). Although speculative, our findings suggest that these benefits of intermittent negative pressure stimulus on wound healing (26, 28) may be related to enhanced endothelial function.

A final possible explanation for our findings relates to the impact of intermittent negative pressure on changes in the pressure gradient across the artery wall (24) and, therefore, transmural pressure (20). Although changes in transmural pressure may affect vascular health (1, 12), it seems unlikely this can explain our findings. First, negative pressure likely increases transmural pressure (due to the drop in external pressure), which is typically associated with impaired vascular health (1). Second, vascular function was examined in the brachial artery, i.e., not directly exposed to the changes in (transmural) pressure, and we observed no significant systemic effects on blood pressure of unilateral forearm suction.

The clinical relevance of our findings is that fluctuations in blood flow or shear rate per se represent a hemodynamic stimulus capable of improving vascular health. Previous studies manipulating shear rate have increased mean shear rate to

improve FMD. In contrast to these stimuli, we have not changed mean shear rate, but still found improved FMD, most likely due to the fluctuations in shear and blood flow patterns. Furthermore, these fluctuations in blood flow and shear rate may be more ecologically valid compared with sustained increases in shear rate. More specifically, fluctuations in blood flow and shear rate are more related to activities of daily living, such as those associated with low-intensity physical activity and changes in posture. Therefore, repetitive exposure to these stimuli may be efficient in improving vascular health. Indeed, recent work has demonstrated that regular exposure to mild physical activity stimuli, such as walking breaks (8, 33) or fidgeting (18), prevents decline in cerebro- and cardiovascular health associated with prolonged sitting. Although speculative, activity-induced fluctuations in blood flow may be the underlying mediator contributing to the preserved vascular health.

Limitations. The present study possesses several strengths, including strict adherence to contemporary expert-consensus guidelines for FMD (29) and blinded data analysis using custom-designed edge-detection software to eliminate operator bias. There are some limitations to the study. First, we recruited healthy recreationally active men, which makes it difficult to extrapolate our findings to other populations (e.g., women) (7, 16, 37) or clinical groups. However, larger improvements in FMD may be observed in those with a priori endothelial dysfunction (17). A second limitation is that we did not perform additional measurements such as blood analysis for markers of endothelial cell activity. In vitro studies in cultured endothelial cells and isolated arteries, reviewed elsewhere (12), demonstrate the release of pro- and antiatherogenic substances in response to exposure to oscillatory (or low) and laminar (or high) shear stress, respectively. Insight into the impact of fluctuations in shear stress (with preserved mean shear) would have contributed to further understanding the underlying mechanisms of our findings. A final limitation relates to the relatively small sample size of our study. Post hoc statistical power analysis using G*Power software (9) revealed a power of 0.77 to detect within-subject changes in FMD, but a power of 0.27 to find a significant interaction effect. Therefore, our results should be interpreted with caution, and further work is required to better understand the potency of fluctuations in shear rate patterns on vascular function.

Conclusions

In conclusion, our findings suggest that 30-min exposure to fluctuations in shear rate improves endothelial function, despite the absence of concomitant changes in mean shear rate compared with resting baseline levels. Our work implies that fluctuations in blood flow or shear rate may represent a hemodynamic stimulus to potentially improve vascular health. Future research to examine the underlying mechanisms and potential long-term effects would be of interest.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.M.H., E.A.D., J.H., D.J.G., and D.H.J.T. conceived and designed research; S.M.H., E.A.D., and Á.B. performed experiments; S.M.H. analyzed data; S.M.H., E.A.D., and D.H.J.T. interpreted results of experiments; S.M.H. prepared figures; S.M.H., E.A.D., D.J.G., and D.H.J.T. drafted manuscript; S.M.H., E.A.D., Á.B., J.H., D.J.G., and D.H.J.T. edited and revised manuscript; S.M.H., E.A.D., Á.B., J.H., D.J.G., and D.H.J.T. approved final version of manuscript.

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