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Reproducibility of post-occlusion reactive hyperaemia assessed by laser Doppler flowmetry in young healthy women

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List of abbrevations:

LDF – laser Doppler flowmetry
PORH – post occlusive reactive hyperaemia
TtP – time to peak
LT – latency time of the flow to go back to the baseline level
AUC – area under the curve
PU – perfusion units
R – vascular reperfusion
ΔR-O – difference between % of flow change during reperfusion and occlusion in relation to baseline
CV – coefficient of variation
ICC – intraclass correlation coefficient
SD – standard deviation

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Abstract

The aim of this study was to evaluate the day-to-day and inter-subject reproducibility of post occlusive reactive hyperaemia (PORH) measured by laser Doppler flowmetry (LDF) in young healthy female subjects. In addition, we looked for most reproducible form of data presentation.

10 healthy female subjects were tested 4 times during 2 weeks. Blood flow was measured during baseline, vascular occlusion (1, 2 and 3 min) and PORH. Parameters studied were: increase in flow during vascular reperfusion (R), difference between % of flow change during reperfusion and occlusion (delta R-O) in relation to baseline, time-to-peak (TtP) and latency time of the flow to go back to baseline levels (LT). Coefficient of variation (CV; with 95% confidence interval) and intraclass correlation coefficient (ICC; with 95% confidence interval) were calculated for each parameter. CVs of ≤10%, 10–25% and ≥25% were considered good, moderate and poor reproducibility. An ICC value of <0.40, 0.40–0.75 and >0.75 considered as poor, fair-to-good and excellent reproducibility.

None of the parameters showed significant differences across the measurements. Obtained CVs for all parameters have shown moderate reproducibility (10-25%), except CV for TtP which was >25%. R-O was the most reproducible parameter of reactive hyperemia, indicated by the lowest CV and highest ICC (0.60), which is considered fair-to-good reproducibility.

The results of this study suggest that parameter R-O is fair-to-good reproducible for LDF assessment of PORH in young healthy women and it can be a useful and reliable noninvasive clinical measurement index of microvascular function.

INTRODUCTION

Laser Doppler flowmetry (LDF) is a non-invasive diagnostic method for monitoring red blood cell flow. LDF enables the evaluation of cutaneous microvascular blood flow and therefore has been widely used to assess cutaneous microcirculation usually coupled with certain reactivity tests *(1)*. Skin has been used as a model of microcirculation in studying vascular disfunctions in certain diseases, including hypertension, diabetes and many cardiovascular risk factors, as well as skin-affected diseases like systemic sclerosis, wounds, burns or flaps *(2)*. Combining LDF with post-occlusive reactive hyperaemia (PORH) provides simple, non-invasive method for examining microvascular and endothelial function *(3)*. PORH refers to the increase in skin blood flow caused by brief arterial occlusion *(1)*. The data in PORH test can be expressed in many different ways, such as time to peak (TtP), latency time of the flow go back to the baseline level (LT), raw area under the curve (AUC) values of the baseline, occlusion and reperfusion *(4)*. Whereas the laser Doppler perfusion signal is a relative measure of flow, standardization is required in comparing perfusion levels in different measurements and from different instruments *(5)*. The duration of occlusion, baseline skin temperature and site of measurement can influence PORH amplitude and reproducibility. LDF has often been considered as poorly reproducible method and major source of variations is the spatial heterogenity of the skin blood flow *(6)*. The intrasubject variability is related mostly to the site of measurement rather than to day-to-day variability *(1)*. Therefore, measuring blood flow on the same place of the forearm could improve reproducibility. Toward to the expression of data in terms of AUC provides more inter- and intra-subject variability than does the expression of data using other parameters, a reliable and universal parameter may increase reproducibility. Another issue when comparing protocols that use PORH is the heterogenity of study protocols. Indeed, there is no consensus about the optimum protocol, and a wide variety in the duration of brachial artery occlusion exists, from 1 to 15 minutes *(7)*. Application of duration-adequate arterial occlusion also might improve repeatability of LDF measurements.

The aim of this study was to evaluate the day-to-day and inter-subject reproducibility of LDF technique on the forearm using PORH test in group of young healthy women by aplying the laser probe on the same spot of the forearm every measurement. Second objective was to investigate most reproducible form of data presentation toward improving reproducibility of the method, and to test new parameter of PORH, ΔR-O which present the difference between percentage of flow change during reperfusion and occlusion in relation to the baseline.

METHODS

Study population

The study was preformed on 10 young healthy female (20±1 years) medical students recruited by advertisment at the Faculty of Medicine University of Osijek participated int he study. Exclusion criteria included a history of hypertension, coronary artery disease, diabetes, hyperlipidemia, renal impairment and peripheral artery diseases. No subjects were taking any medications that could affect their cardiovascular system. Written informed consent was obtained from each subject. The study protocol and procedures conformed to the standards set by the latest revision of Declaration of Heslinki and were approved by the Ethical Commitee of the Faculty of Medicine, University of Osijek. Volunteers were randomised considering menstrual cycle's phase to avoid potential influence of sex hormones on microcirculation blood flow.

Study protocol

Each subject was studied four times during two weeks. The testing occured at approximately the same part of the day. Upon arrival, subjects were placed in a temperature controlled room (23±1 °C) where they spent a 30 min acclimatization period. The subjects were set in the supine position during the testing. The cushion was used to decrease artifacts associated with arm movements. The testing skin point was elected on the ventral side of the right forearm, more than 5 cm from the wrist, avoiding visible veins. The eligibility norm for choosing adequate point was that flux value amounts around 5 PU (perfusion units). Laser Doppler flowmetry was applied on the same place of the forearm every single measurement. Testing consisted of 3 occlusion intervals which were induced by inflating a cuff placed on the right arm to 50 mmHg above the systolic blood pressure. First occlusion interval lasted 1 min, second interval lasted 2 min and the third lasted 3 min. Among every occlusion interval, baseline section was preformed and recorded in duration of 10 min. Blood preasure and heart rate were recorded before every measurement.

Data analysis

Data were digitized, stored on a computer and analyzed off-line with signal processing software (MoorVMS-PC, Moor Instruments Ltd Millwey Axminister, UK). Measured parameters were increase in flow during reperfusion following vascular reperfusion (R), time-to-peak (TtP), latency time of the flow to go back to the baseline levels (LT) expressed as raw area under the curve (AUC) values and difference between percentage of flow change during reperfusion and occlusion in relation to the baseline (ΔR-O) (Figure 1).

Statistical analysis

The values obtained for the different parameters are presented as the mean and standard deviation for each measurement. Inter-day reproducibility was carried out using within-subject coefficient of variation (CV), with 95% confidence interval. The reliability of measurements was assessed using intraclass correlation coefficient (ICC), with 95% confidence interval. The CV values were calculated as ratio of the standard deviation (SD) to the mean. CVs of ≤10%, 10-25% and ≥ 25% were regarded as good, moderate and poor reproducibility. The ICC values with $<$0.40, 0.40-0.75 and $>$0.75 were considered as poor, fair-to-good and excellent reproducibility.

Data were stored and transformed using Microsoft Excel (Microsoft Office 2007, Microsoft Corporation,

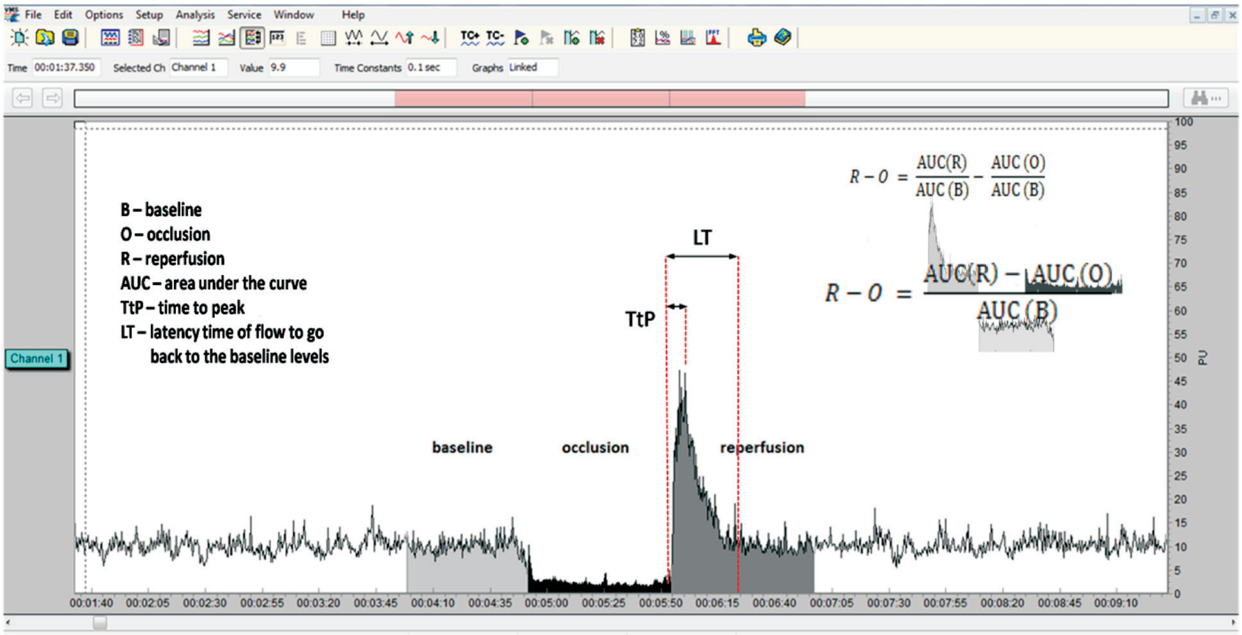


Figure 1. *Laser Doppler Flowmetry (LDF) measurement of skin microvascular blood flow. Microcircularory blood flow in a given time was determined by program calculating area under the curve (AUC) during baseline flow, occlusion and reperfusion (AUC is denoted by shaded portions of the trace). Final result was expressed as the difference between the percentage of flow change during reperfusion and occlusion (delta R-O) in relation to baseline. Other measured parameters were time-to-peak (TtP) and latency time of the flow to go back to the baseline levels (LT). (Source: original trace, Laboratory for Vascular Physiology Department of Physiology and Immunology Faculty of Medicine Osijek).*

Redmond, WA) and statistical analyses were performed using IBM SPSS Statistics v20.0 (IBM Corporation, New York, USA).

RESULTS

This study included 10 young healthy female subjects. Participant characteristics are presented in Table 1. There were no statistically significant differences in blood pressure, pulse or skin temperature among subsequent measurements. Studied parameters, including raw AUC values of the baseline and occlusion are presented in Table 2. There were no significant within- or inter- subject differences between baseline measurements on each of the study days (Figure 3).

The inter-day reproducibility and the reliability of measurements using CV and ICC are presented in the Table 3.

Coefficients of variation

The CV values of R ranged between 16-23% are indicating moderate reproducibility. CV values for TtP (13-40%) and LT (18-37%) showed poor reproducibility. The CV values for ΔR-O parameter (13-19%) showed moderate reproducibility and demonstrated ΔR-O as the most reproducible parameter compared to the other parameters measured in the study.

TABLE 1

Participant characteristics.

Age (years)	20 ± 1
Body mass (kg)	60 ± 6
Stature (cm)	169 ± 4
Systolic blood pressure	110 ± 9
Diastolic blood pressure	71 ± 6
Heart rate	83 ± 15
Skin temperature (°C)	30 ± 1

*n=10 subjects
*data are expressed as mean ± SD

The CV values observed as 1, 2 and 3 min vascular occlusion indicates that 1 min (16-29%) and 3 min (13-37%) vascular occlusion test are moderate reproducible while 2 min vascular occlusion test (19-40%) showed poor reproducibility (Figure 2).

Intraclass correlation coefficients

The ICC data of ΔR-O in range of 0.44-0.76 indicated its fair-to-good reproducibility and marked ΔR-O as the most reproducible parameter according to the reliability of measurements when compared with ICC values of R (-0.50-0.41) and TtP (-0,09-0.65) that have shown

TABLE 2
Measured parameters.

		AUC	R-O	TM	TL
	Baseline	397,43 (52,87)			
1 min	Occlusion	180,88 (29,23)	1,28 (0,24)	6,15 (1,47)	43,125 (5,67)
	Reperfusion	686,17 (114,24)			
	Baseline	786,94 (108,56)			
2 min	Occlusion	347,30 (66,29)	1,30 (0,25)	9,25 (1,96)	75,25 (15,20)
	Reperfusion	1352,0 (314,5)			
	Baseline	1164,73 (221,74)			
3 min	Occlusion	515,91 (88,37)	1,37 (0,18)	11,95 (2,01)	116,8 (18,04)
	Reperfusion	2106,12 (447,33)			

*data are expressed as mean (SD)
*n= 10 subjects
*N= 4 measurements per each subject

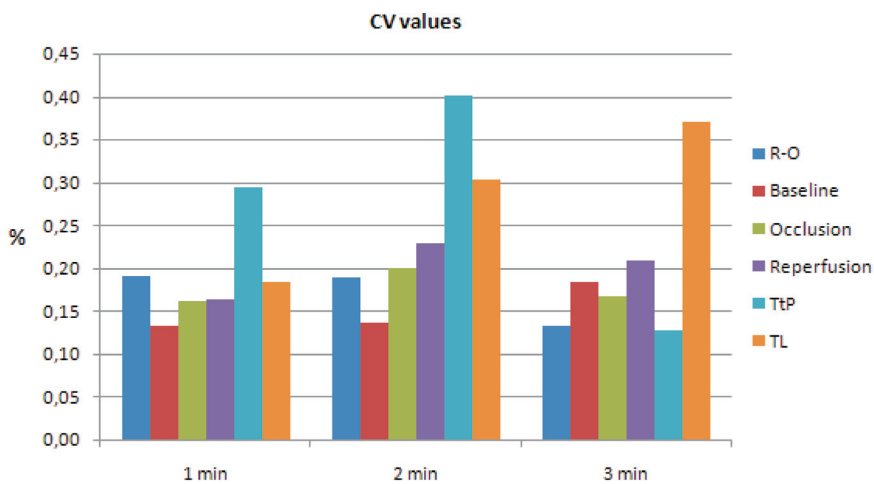


Figure 2. ***ΔR-O parameter and 3 min vascular occlusion test showed the best reproducibility.*** *Coefficient of variation (CV) is the ratio of the standard deviation (SD) to the mean. CVs of ≤10%, 10-25% and ≥ 25% were regarded as good, moderate and poor reproducibility. CV values for TtP and LT showed poor reproducibility. The CV values for ΔR-O parameter (13-19%) showed moderate reproducibility and demonstrated ΔR-O as the most reproducible parameter compared to the other parameters measured in the study. The CV values observed as 1, 2 and 3 min vascular occlusion indicates that 3 min (13-37%) vascular occlusion test is most reproducible.*

negative and are interpreted as indicating no agreement. The ICC data of LT (0.16-0.55) indicated poor reproducibility of the parameter.

Obtained ICC data for ΔR-O parameter observed through 1, 2 and 3 min vascular occlusion indicated 3 min vascular occlusion test as excellent reproducible method with ICC 0.76.

DISCUSSION

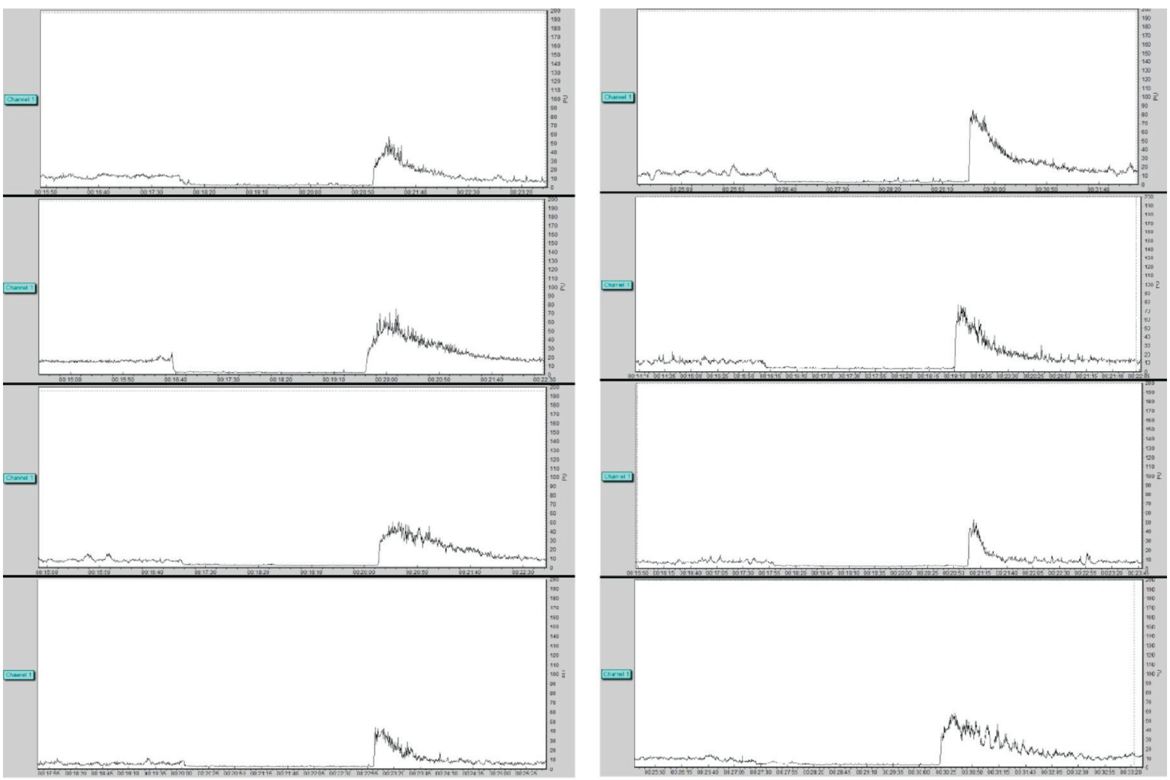
The present study provides results about inter-subject and within-subject reproducibility of PORH asessed by LDF technique in order to assess microvascular function in clinical routine. Considering that the skin's microcirculation composed of capillaries, arterioles, venules and arteriovenous anastomosis is a vital aspect of normal thermoregulation in humans, variations in ambient or local temperature leads to large differences in cutaneous vascular blood flow and frequently is considered as the major reason of LDF regarded poorly reproducible. Recent studies showed PORH reproducibility decidedly poor when the skin sites of measurement were randomly chosen or with less precision *(3)*. Moreover, intrasubject variability is related mostly to the site of the measurement rather than day-to-day variability *(8)*. Applying laser probe on the same spot on the forearm during measurement contributed to the improvement of the reproducibility of the method in our study. Reproducibility of the method is

TABLE 3

Reproducibility data for measured parameters.

		R-O	Baseline	Occlusion	Reperfusion	TM	TL
1 min	Mean	1,28	397,43	180,88	686,17	6,50	43,00
	CV	0,19	0,13	0,16	0,16	0,29	0,18
	95% CI	0,24	51,81	28,64	111,95	1,88	7,72
2 min	Mean	1,30	786,94	347,30	1352,00	8,25	74,00
	CV	0,19	0,14	0,20	0,23	0,40	0,30
	95% CI	0,25	106,38	64,96	308,21	3,24	22,00
3 min	Mean	1,37	1164,73	515,91	2106,12	11,75	95,75
	CV	0,13	0,18	0,17	0,21	0,13	0,37
	95% CI	0,18	217,31	86,60	438,37	1,47	34,77
1 min	ICC	0,58	0,69	0,06	0,41	0,504	0,55
	95% CI	-0,02 0,88	0,25 0,91	-1,53 0,74	-0,53 0,83	-0,41 0,87	-0,08 0,87
2 min	ICC	0,44	0,40	0,18	-0,50	0,655	0,163
	95% CI	-0,36 0,84	-0,58 0,84	-1,19 0,78	-2,90 0,58	0,07 0,91	-1,29 0,77
3 min	ICC	0,76	-0,02	-0,23	0,08	-0,099	0,22
	95% CI	0,38 0,93	-0,96 0,73	-1,91 0,64	-1,31 0,74	-1,95 0,70	-0,58 0,75

*n= 10 subjects
*N= 4 measurements per each subject



SUBJECT 1 SUBJECT 2

Figure 3. ***Day-to-day measurement of skin microvascular blood flow.*** *Day-to-day intrasubject evaluation of microvascular blood flow using LDF has shown fair-to-good reproducibility. The data in PORH test can be expressed in many different ways, such as time to peak (TtP), latency time of the flow go back to the baseline level (LT), raw area under the curve (AUC) values of the baseline, occlusion and reperfusion. The expression of data in form of raw AUC values (as can be seen in the picture) provides more inter- and intra-subject variability. Major role in hyperaemic response pertains to the baseline values of the blood flow. Therefore, the difference between the percentage of flow change during reperfusion and occlusion (Δ R-O) in relation to baseline could improve reproducibility of the method.*

also determined by parameters that are used in the data and statistical analysis. Many parameters can be quantified from blood flow and no consensus exists concerning parameter selection. In some recent clinical studies flow response was expressed as a raw value of the peak minus baseline and AUC *(9),* raw value of the peak minus biological zero *(10),* percentage of the baseline value *(11),* and increase in postischemic flow using the AUC at baseline and postischemia *(12).* The expression of data in form of AUC provides more inter- and intra-subject variability than does the expression of data standardized to a maximal vasodilatation *(13).* Prior studies indicated that the PORH reproducibility expressed as CV is poor (40% or higher) when peak perfusion is expressed as a function of a baseline *(14).* Results of measuring and studying many parameters that describe blood flow during this study indicated, new parameter ΔR-O as the most reproducible parameter of reactive hyperaemia indicated by the lowest CV and the highest ICC. Other parameters considered in this study, showing poor or fair-to-good reproducibility, have proven to be less reproducible than the ΔR-O parameter. PORH refers to the increase in skin blood flow above baseline levels following release from brief arterial occlusion and is one of the commonly used reactivity test in examining microvascular function. There is no consensus about which ischemic period to use so it varies among 1, 2, 3, 5, 10 and 15 min., but positive relationship between postocclusive ischemic response and the duration of the arterial occlusion exists *(15, 16).* Applied ischemic periods in this study were 1 min, 2 min and 3 min. According to ICC values, results showed that 3 min occlusion test is the most reproducible method of examining microvacular reactivity in comparison with other periods applied.

Collectively, this findings indicate that the new ΔR-O parameter is fair-to-good reproducible and can be reliably assessed in LDF measurements. Furthermore, PORH, especially 3 min ischemic period showed its utility when examining cutaneous microvascular function. Aplying LDF probe on the same skin site every measurement to avoid cutaneous microvascular spatial heterogenity contributed to the increase in reproducibility of the LDF method. These findings have shown moderate reproducibility. Excellent reproducibility is probably very difficult to accomplish owing to the inability of LDF probes to account for the assumed large spatial heterogenity in forearm capillary density *(17).* We conclude that LDF using PORH reactivity test considering the probe application on the same skin place is useful an reliable noninvasive measurement index of microvascular function that could be used in clinical observations.

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