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Abstract

Tissue occlusion and the hyperaemic response upon reperfusion can be used as a tool to assess microvascular function in various vascular diseases. Currently, laser Doppler flowmetry (LDF) is applied most often to measure hyperaemic responses. In this study, we have applied Tissue Viability Imaging (TiVi) and LDF to measure the change in red blood cell concentration and perfusion in the skin after occlusions of the forearm with increasing duration. We have found that there is a strong correlation between the changes in perfusion and red blood cell (RBC) concentration during post-occlusive hyperaemia (perfusion: r = 0.80; RBC concentration: r = 0.94). This correlation increases with longer occlusion durations (1, 5 and 10 minutes). Furthermore, for both the perfusion and the RBC concentration, the maximum responses (perfusion: r^2 = 0.59; RBC concentration: r^2 = 0.78) and the recovery times (perfusion: r^2 = 0.62; RBC concentration: r^2 = 0.91) increase linearly with the duration of the occlusion. Maximum responses and recovery times were more reproducible for RBC concentration (as measured with TiVi) than for perfusion (as measured with LDF). These results show that perfusion and RBC concentration are related during post-occlusive hyperaemia and that TiVi can be used as a tool in the assessment of hyperaemic responses that has advantages in terms of reproducibility, sensitivity and easy of use.

Keywords: occlusion, hyperaemia, laser Doppler, Tissue Viability imaging, perfusion, red blood cell concentration

Introduction

Post-occlusive reactive hyperaemia (PORH) is the increase of blood perfusion after a period of circulatory arrest that occurs in virtually all major vascular beds. PORH has been studied extensively in the past, and it is thought that the hyperaemic response involves endothelial, myogenic, metabolic, and physical mechanisms (Banitt et al., 1996; Beinder and Schlembach, 2001; Lombard and Duling, 1981)

PORH can be used as an instrument to investigate microvascular function in a variety of vascular diseases. The method has been used to study the effect of diabetes (Yamamoto-Suganuma and Aso, 2009), heart failure (van Langen et al., 2001), peripheral vascular disease (Cheng et al., 2004), preeclampsia, (Beinder and Schlembach, 2001) and cigarette smoking (Hashimoto, 1994).

The vascular response during and after occlusion of the vascular supply can be measured by various techniques. The techniques that have been used include those commonly used with isolated arteries, such as myography (Rogers and Sheriff, 2005) and intravital microscopy (Toth et al., 2007). In human studies, PORH has been measured by plethysmography techniques (Acree et al., 2007; Faizi et al., 2009), ultrasound (van Langen et al., 2001) and laser Doppler flowmetry (Beinder and Schlembach, 2001; Hashimoto, 1994; Thorfinn et al., 2007; Yamamoto-Suganuma and Aso, 2009).

Laser Doppler flowmetry (LDF) may be considered the most widely used method to evaluate PORH in the skin of the forearm. LDF is an easy-to-use, non-invasive technique that yields a measure of perfusion in the cutaneous microvascular bed when it is applied on the skin. Perfusion as measured by LDF is a product of two factors: RBC concentration and RBC velocity (Bonner and Nossal, 1981).

Recently, an alternative to LDF for investigation of the skin microcirculation has been developed, the Tissue Viability Imager (TiVi), which is a device based on polarization light spectroscopy. This technique can 'see through' the top layer of

the skin by use of filters with orthogonal polarization directions. In this way, changes in RBC concentration in the cutaneous microvascular bed can be detected. In contrast to LDF, polarised light spectroscopy provides an instantaneous measurement of a large area of skin, at a reasonably high temporal resolution. The TiVi system has recently been evaluated in separate studies regarding performance (Nilsson et al., 2009), ability to visualize skin blanching (Zhai et al., 2009), and in the assessment of post-occlusive reactive hyperaemia (McNamara et al., 2010).

The duration of the occlusion is known to influence the hyperaemic response. Similarly, area-under-the-curve (AUC) of the hyperaemic response and the time-to-peak response are affected. However, the relation between occlusion duration and response is dependent on the tissue under study and on the measurement technique. In most animal and human studies, longer occlusion times have been found to result in responses that are larger in magnitude. However, it has also been suggested that the hyperaemic response reaches a maximum with 5 minutes of occlusion (Faizi et al., 2009; Thorfinn et al., 2002; Thorfinn et al., 2007; Yvonne-Tee et al., 2008). For clinical assessment of vascular function, it has been suggested that 3 minutes (with LDF) or 5 minutes (with plethysmography) is the optimal occlusion duration, as those seem to result in maximal hyperaemic responses (Faizi et al., 2009; Yvonne-Tee et al., 2008). Moreover, the discomfort for the subjects increases with occlusion duration.

It is important to further compare the TiVi-technique to LDF, which is considered the gold standard when measuring PORH. Therefore, the aim of this study was to investigate and compare the change in perfusion (with LDF) and in red blood cell concentration (with TiVi) with increasing occlusion durations (1, 5, and 10 minutes respectively) in the cutaneous microvascular bed of the forearm.

Methods

Subjects

9 healthy male volunteers with a mean age of 27.3 (2.7) were included in the study after they had given informed and written consent. They were asked not to drink alcoholic drinks or coffee or tea for 2 hours before the experiments. All subjects were normally active. Reasons for exclusion were cardiovascular disease, diabetes, skin diseases, regular use of nicotine, intake of medications (except oral contraceptives), systolic blood pressure > 150 mmHg and/or diastolic blood pressure > 90 mmHg. Blood pressure was measured before the start of the experiments. The study was done according to the Declaration of Helsinki and was approved by the medical ethics committee at Linköping University, Sweden.

Laser Doppler flowmetry

A PeriFlux system 5000 Laser Doppler Perfusion Monitoring unit (Perimed AB, Järfälla, Sweden) with a thermostatic laser Doppler probe (Probe 481-1, Perimed AB, Järfälla, Sweden) was used to measure changes in skin perfusion and skin temperature (Bonner and Nossal, 1981). The bandwidth of the system was 15 kHz. The system was calibrated before the start of the study according to the guidelines of the manufacturer. The probe used in the current study has a fibre separation of 0.25 mm and collects perfusion data at a depth of about 0.5-1 mm.

Blood flow data were recorded continuously during the experiments at a sample rate of 32 recordings per second, and were later averaged over 10-second periods, to match the sampling rate of the TiVi system.

Data from the laser Doppler perfusion monitor were analysed using PeriSoft for Windows, version 2.5.5 (Perimed AB, Järfälla, Sweden).

Tissue Viability Imaging

A commercial system (TiVi600, WheelsBridge AB, Linköping, Sweden), based on subsurface polarisation light spectroscopy (Nilsson et al., 2009) was used to

measure changes in RBC concentration in the skin. The system consists of a digital camera equipped with perpendicular polarisation filters in front of the flash and lens. When the flash is activated, the broad-spectrum white light that is emitted becomes linearly polarised after it has passed the first polarisation filter. Reflected light from the skin consists of both polarised (directly reflected) and randomly polarised ("subsurface") light. A second filter in front of the lens prevents any directly reflected light from reaching the photo-array in the camera. The RBCs in the microcirculation absorb light in the green wavelength region (about 500–600 nm) to a much higher extent than light in the red wavelength region (about 600–700 nm). In comparison, the surrounding tissue components of the dermis absorb lesser light, and this absorption is not as wavelength dependent as that of the RBCs. The TiVi-technology takes advantage of this difference in absorption by separating the images into their different colour planes.

An algorithm subtracts the value of each picture element in the green colour matrix from the corresponding value in the red colour matrix, and divides the result by a signal proportional to the total light intensity within the actual wavelength region. The values of the resulting matrix, referred to as TiVi-indices, scale linearly with the momentary concentration of RBCs in the tissue volume. The system is relatively insensitive to the oxygen state of the RBCs as confirmed by in vitro experiments demonstrating a reduction in TiVi-index limited to about 10% when saturation was reduced from 100 to 0% (O'Doherty et al., 2007).

The TiVi-system was positioned 15 cm above the volar aspect of the forearm on which the laser Doppler probe was placed. The TiVi camera resolution was set to "small fine" (640x480 pixels), and the settings "flash on" and "macro" were used. Photographs were captured with 10-second intervals 1 minute before occlusion (baseline), during occlusion, and 15 minutes after release of the pressure cuff.

Image analysis was made using the TiVi analysis software (TiVi Version 2.1, WheelsBridge AB, Linköping, Sweden). The images contained the complete volar aspect of the lower forearm, from the elbow to the wrist. An average TiVi-index

was calculated for each image, which resulted in a recording of the change in red blood cell concentration over the time course of the experiment.

Protocol

Ambient conditions (temperature 23 (1) °C and room humidity 30%) were kept as constant as possible throughout the experiment. Subjects were resting comfortably in a supine position and were asked to move their arms as little as possible during the experiments. Pillows were used to support the arms at heart level. A pressure cuff was applied around the upper arm. After cleaning the skin of the volar aspect of the forearm, the laser Doppler probe was applied to the skin using double adhesive tape and the TiVi camera was positioned above the area where the probe was located. Care was taken to choose a position free from superficial veins and skin irregularities. An acclimatisation period of 20 minutes was used before the start of the measurements.

After a baseline measurement of 3 minutes, local ischemia was induced in the forearm by inflating the pressure cuff to a suprasystolic pressure of 250 mmHg. Inflating the cuff took less than 3.3 seconds. In the first test, blood flow was stopped for 1 minute, in the second for 5 minutes and in the last test for 10 minutes. The hyperaemic responses were measured for 15 minutes after the release of the pressure. Deflating the cuff was practically instantaneous (< 40 mmHg within 0.2 seconds). Tests were separated by a resting period of 10 minutes (Table 1).

Statistics

Baseline for both LDF and TiVi was defined as the average value of the 3-minute baseline period before occlusion. Maximum response was defined as the maximum absolute change from baseline in arbitrary units (A.U.). Recovery time was defined as the time in seconds needed for the perfusion to decrease from the maximum value to 50% of the maximum value. Area under the hyperaemic curve (AUC), also in arbitrary units, was calculated from the time the cuff was released until the end of the measurement, 15 minutes later. Data is presented as mean (S.D.) and variability was assessed using coefficients of variation (%CV). Baseline

data for different occlusion durations were compared using 1-way ANOVA for repeated measures. Differences in recovery time between LDF and TiVi, and between occlusion times, were compared using 2-way ANOVA for repeated measures. Bonferroni post-tests were used for comparison between LDF and TiVi at specific occlusion durations. Statistical calculations were done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, "www.graphpad.com"). For all analyses, probabilities of less than 0.05 were accepted as significant.

Results

Neither a 1-way ANOVA nor a linear regression was able to show a significant variation in baseline values during the first minute of baseline measurement, indicating that the vasculature was adequately stabilised after 20 minutes of acclimatisation. Numerical results for the perfusion and RBC responses after 1, 5, and 10 minutes of occlusion are shown in Table 2.

Perfusion (Laser Doppler flowmetry)

Baseline perfusion was similar for different occlusion durations (1 minute, 5.3 (2.5) A.U.; 5 minutes, 6.1 (3.0) A.U.; 10 minutes, 5.5 (4.0) A.U.; p = 0.87).

During occlusion, a quick decrease in perfusion was observed. After release of the pressure cuff, an instant increase in perfusion (1 minute, 7.1 (3.4) A.U.; 5 minutes, 14.0 (5.0) A.U.; 10 minutes, 23.2 (8.0) A.U.), followed by a gradual decrease in perfusion was found for all occlusion durations (Figure 1). The recovery time, defined as the time needed for the perfusion to decrease from the maximum value to 50% of the maximum value, was 16 (7) s for 1 minute occlusion, 48 (16) s for 5 minutes occlusion and 137 (68) s for 10 minutes occlusion. The area under the hyperaemic curve was 866 (601) s for 1 minute occlusion, 1991 (824) s for 5 minutes occlusion and 4115 (1292) s for 10 minutes occlusion.

Linear regression analysis showed that maximum hyperaemic perfusion response, recovery time and AUC were linearly proportional to the occlusion duration ($R^2 = 0.59$, $R^2 = 0.62$ and $R^2 = 0.68$, respectively, $p < 0.001$).

At the end of the 15-minute period after release of the pressure cuff, perfusion had returned to baseline level for all occlusion durations (Paired Student's t – test; no significant difference from baseline).
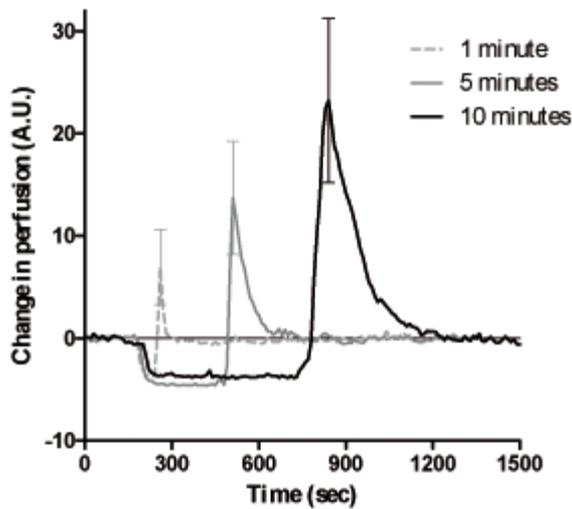
Figure 1. Change in perfusion in the forearm skin before, during, and after arterial occlusion for 1 minute (dotted line), 5 minutes (light grey line), and 10 minutes (black line) as measured by laser Doppler flowmetry. Maximum responses and recovery times increased linearly with occlusion duration ($R^2 = 0.59$ and $R^2 = 0.62$, respectively; $p < 0.001$). *Error bars indicate ± 1SD.*

RBC concentration (Tissue Viability Imaging)

Baseline RBC concentration was similar for different occlusion durations (1 minute, 117 (28) A.U.; 5 minutes, 115 (30) A.U.; 10 minutes, 117 (31) A.U.; p = 0.98).

During occlusion, a minor decrease in RBC concentration was observed only during the longest occlusion duration of 10 minutes. After release of the pressure cuff, an instant increase in RBC concentration (1 minute, 32 (10) A.U.; 5 minutes, 73 (14) A.U.; 10 minutes, 101 (19) A.U.) was found for all occlusion durations, followed by a gradual decrease in RBC concentration (Figure 2). The recovery time was 26 (7) s for 1 minute occlusion, 103 (16) s for 5 minutes occlusion and 177 (29) s for 10 minutes occlusion. The area under the hyperaemic curve was 3799 (1174) s for 1 minute occlusion, 13141 (6068) s for 5 minutes occlusion and 24680 (9651) s for 10 minutes occlusion.
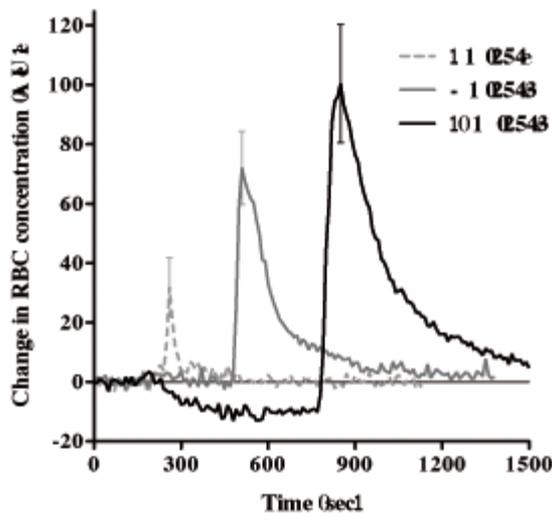
Figure 2. Change in red blood cell concentration in the forearm skin during, and after arterial occlusion for 1 minute (dotted line), 5 minutes (light grey line), and 10 minutes (black line) as measured by Tissue Viability Imaging. Maximum responses and recovery times increased linearly with occlusion duration ($R^2 = 0.78$ and $R^2 = 0.91$ respectively, p < 0.001) *Error bars indicate ± 1SD.*

Linear regression analysis showed that the amplitude of the hyperaemic RBC concentration responses and recovery time were linearly proportional to the occlusion duration ($R^2 = 0.78$, $R^2 = 0.91$ and $R^2 = 0.65$, respectively, p < 0.001).

At the end of the 15-minute period after release of the pressure cuff, RBC concentration had returned to baseline level for all occlusion durations (Paired Student's t – test; no significant difference from baseline).

Perfusion compared with RBC concentration

There was a significant difference in recovery time between perfusion (LDF) and RBC concentration (TiVi) for 5 minutes occlusion (49 (16) and 103 (16); p < 0.01) and 10 minute occlusion (137 (68) and 177 (29); p < 0.05).

A strong correlation between perfusion and red blood cell concentration was found, both when individual data was considered and when data was averaged (Figure 3). The strength of the correlation was dependent on the occlusion time. For individual data, Pearson's correlation coefficients were 0.28, 0.68 and 0.84 for 1 minute, 5 minutes and 10 minutes occlusion, respectively. The overall correlation for all occlusion durations was 0.80 (Figure 3). For pooled data, correlation coefficients were 0.8, 0.92 and 0.97 for 1 minute, 5 minutes and 10 minutes occlusion, respectively. The overall pooled correlation for all occlusion durations was 0.94.
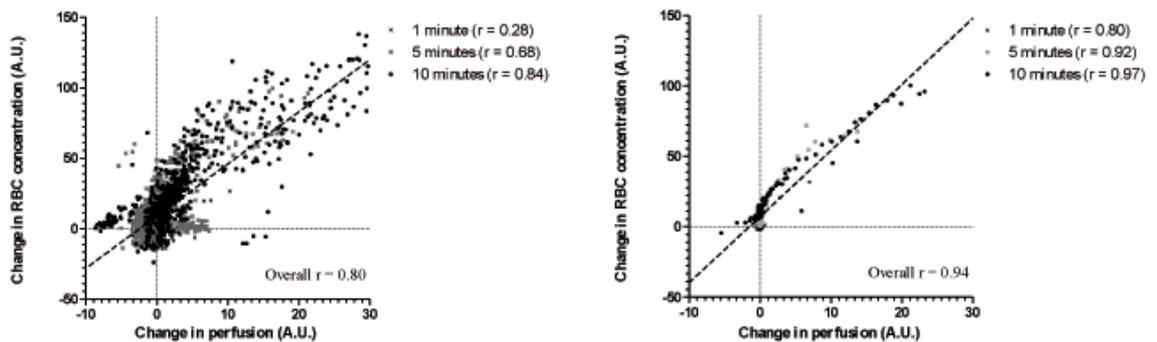

Figure 3. Correlations between perfusion (as measured with laser Doppler flowmetry) and red blood cell concentration (as measured with Tissue Viability Imaging) for post-occlusive hyperaemic responses after increasing durations of arterial occlusion. Left graph shows data points for each subject separately, right graph show data as mean values of all subjects at each time point.

Discussion

In this study we have shown that the hyperaemic response after occlusion of the forearm (PORH) of healthy male volunteers depends on the duration of the occlusion for durations between 1 minute and 10 minutes. Our results suggest that TiVi can be used as an alternative technique to laser Doppler flowmetry in the assessment of PORH in the skin since the two measures were strongly correlated. This may be important in clinical situations were accurate and fast registration of skin blood perfusion is important, such as during monitoring of free flaps in reconstructive surgery (Abdel-Galil and Mitchell, 2009; Smit et al., 2010). However, it should be noted that the results obtained by these methods are different entities. TiVi measures the presence of RBCs in the vascular bed of the skin (RBC concentration), whereas LDF measures the perfusion (concentration of RBCs multiplied by the average velocity of the RBCs).

In contrast to the findings of Faizi et al. (2009), who used finger plethysmography, we found that when using LDF and TiVi, 10 minutes occlusion time results in significantly stronger responses compared with shorter occlusions, both for hyperaemic amplitude, area-under-curve and recovery time. On the other hand, the reproducibility of the responses after 5 minutes of occlusion was at least as good as after 10 minutes of occlusion with LDF and with TiVi (although not for area-under-curve). Although 10 minutes of occlusion was well tolerated by all the subjects in the study and stronger responses were observed, there seems to be a benefit of using 5 minutes of occlusion compared to 10 minutes regarding measurement time and discomfort.

Following occlusion of the blood flow to a limb, the TiVi-index did not approach a "biological zero" (Kernick et al, 1999) in the same way as the perfusion value recorded by laser Doppler techniques, because the TiVi-system is sensitive only to the RBC concentration and not to the RBC velocity. The slight decrease in TiVi-index during the 10 minutes occlusion may be due to a lower oxygen saturation of the blood, although the TiVi-index should be rather independent to changes in

oxygen saturation as determined in vitro, with a decrease in TiVi-index of at most 10% when oxygen saturation decreases from 100% to 0% (O'Doherty et al., 2007). During complete arterial occlusion, RBC concentration in the forearm cannot change. However, there could very well be a redistribution of RBCs in the vascular bed when the arterial supply to the vascular bed is halted. This may explain a decrease in measured RBC concentration, for instance when part of the RBCs move towards deeper laying regions in the vascular bed that are outside the measurement volume of the TiVi system.

In the current study, we find that the variability in responses between subjects differs substantially between the different response measures and between the occlusion times. Maximum response and recovery time show roughly comparable variability, but lower than the variability of the area under the hyperaemic curve, particularly with TiVi. The variability of the TiVi responses between subjects was much lower compared to LDF for baseline, maximum response, and recovery time. This is most likely the result of the large area over which the RBC concentration was measured. The TiVi-index is an average value obtained from thousands of image pixels from the volar aspect of the forearm, while the LDF signal is measured on a single skin site with a measurement radius of typically a few millimetres. Even though this can be considered an advantage of TiVi, it should be noted that averaging of a large area might obscure regional effects and possibly also affect the measurement of response dynamics. For example, the slower recovery time as measured by TiVi may be caused by the fact that proximal and distal areas are averaged into a single figure. Further studies may clarify how responses in RBC concentration might differ between proximal and distal sites.

The variability in responses between subjects may be partly caused by variations in the transport of cuff pressure to the blood vessels. Occlusion of a smaller tissue volume, such as when using a finger cuff, may enhance the reproducibility of the responses, since pressure changes are more rapidly transmitted to the vessels. However, since upper arm occlusion is much more common procedure

than finger occlusion, we chose to use the most common occlusion technique in the current study.

We found that RBC concentration and perfusion during post-occlusive hyperaemia as measured by the two techniques were strongly related, and that the correlation strengthens with occlusion time. It is likely that this dependency on occlusion time is caused by the fact that longer occlusions give stronger responses. Stronger responses yield a broader range of measurement values, resulting in more well-defined correlations and hence higher correlation coefficients. We present both correlations based on individual data and on pooled data. The former gives insight in how RBC concentration and perfusion correlate within individual subjects, and the latter how the results obtained with the two techniques correlate at group-level.

From the correlation plots it can be seen that at low perfusion values, the changes in RBC concentration are larger than the changes in perfusion. This indicates that TiVi is more sensitive in the low perfusion range, which agrees with previous studies (Henricson et al., 2009). At increased perfusion values, the relation between the two measures seems to be more or less linear, although it is hard to draw strong conclusions about the exact nature of the relation based on the current data. Further investigations could be focussed on exploring this relation in more detail. The overall correlation coefficient of 0.80 for individual and 0.94 for pooled data indicates that the hyperaemic response consists of a concomitant change in RBC concentration and perfusion.

There are some limitations with this study. We only investigated healthy men, and therefore the results cannot be extrapolated to a general population. It has been shown in a previous study that maximum responses are influenced by gender and menstrual cycle (Yvonne-Tee et al., 2008). We did not randomize the order in which the occlusion durations were applied, and therefore we cannot completely rule out the possibility that previous occlusions have affected the responses. However, to overcome a possible systematic error because of this, we waited at least 25 minutes between the occlusions (15 minutes measurement and 10 minutes rest). This was likely to have the desired effect since in all

subjects, blood flow and RBC concentration values had returned to baseline at the start of a new occlusion.

Although TiVi is clearly able to detect the rapid increase in RBC concentration after release of the pressure cuff, the maximum RBC concentration responses may be underestimated as a result of the limited temporal resolution of 1 measurement per 10 seconds. The fact that the TiVi-index is a measure averaged over a large area will also affect the maximum responses.

To summarise, we found that in the skin of the forearm, the maximum perfusion and RBC concentration in the hyperaemic phase increase linearly with occlusion durations up to 10 minutes. This study shows for the first time how skin perfusion and RBC concentration for different occlusion durations (1, 5, and 10 minutes) correlate during occlusion and during post-occlusive hyperaemia. Moreover, we conclude that Tissue Viability Imaging offers an interesting alternative to conventional methods to assess post-occlusive hyperaemia, such as plethysmography and laser Doppler flowmetry. The technique is easy to use and offers higher sensitivity than LDF at low blood flows. Laser Doppler flowmetry and Tissue Viability Imaging can be used together in future studies to further investigate the relation between perfusion and RBC concentration in post-occlusive reactive hyperaemia and in other vascular provocations. This may increase the knowledge about underlying vascular mechanisms or (patho-)physiological conditions. Finally, additional studies are needed to fully assess the advantages of TiVi in terms of reproducibility in the measurement of post-occlusive hyperaemia.

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Tables

Table 1

Table 1. The protocol used for studying the hyperaemic response in the forearm skin of healthy subjects after arterial occlusion for 1, 5, and 10 minute using laser Doppler flowmetry (LDF) and Tissue Viability Imaging (TiVi).

	Acclimatisation	**Baseline**	**Occlusion**	**Post-occlusion**	**Rest**
Test 1	20 minutes	3 minutes	1 minute	15 minutes	10 minutes
LDF		continuous measurement			-
TiVi	-	18 images	6 images	90 images	-
Test 2	-	3 minutes	5 minutes	15 minutes	10 minutes
LDF		continuous measurement			-
TiVi	-	18 images	30 images	90 images	-
Test 3	-	3 minutes	10 minutes	15 minutes	-
LDF		continuous measurement			-
TiVi	-	18 images	60 images	90 images	-

Table 2

Perfusion and red blood cell concentration before, during and after 1, 5, and 10 minutes of arterial occlusion. Perfusion was measured with laser Doppler flowmetry (LDF) and red blood cell concentration was measured with Tissue Viability Imaging (TiVi).

| | **Baseline (A.U.)** | | | | | |
| | *Perfusion* | | | *RBC concentration* | | |
Duration (min)	Mean	SD	%CV	Mean	SD	%CV
1	5.3	2.5	47%	117	28	24%
5	6.1	3	49%	115	30	26%
10	5.5	4	73%	117	31	26%

| | **Maximum response (A.U.)** | | | | | |
| | *Perfusion* | | | *RBC concentration* | | |
Duration (min)	Mean	SD	%CV	Mean	SD	%CV
1	7.1	3.4	48%	32	10	31%
5	14	5	36%	73	14	19%
10	23	8	35%	101	19	19%

| | **Speed of recovery (seconds)** | | | | | |
| | *Perfusion* | | | *RBC concentration* | | |
Duration (min)	Mean	SD	%CV	Mean	SD	%CV
1	16	7	44%	26	7	27%
5	49[**]	16	33%	103[**]	16	16%
10	137[*]	68	50%	177[*]	29	16%

| | **Area under hyperaemic curve (A.U.)** | | | | | |
| | *Perfusion* | | | *RBC concentration* | | |
Duration (min)	Mean	SD	%CV	Mean	SD	%CV
1	866	601	69%	3799	1174	31%
5	1991	824	41%	13141	6068	46%
10	4115	1292	31%	24680	9651	39%

* Significant difference between Perfusion and RBC concentration, p < 0.05; ** p < 0.01.