Title: A Systematic Evaluation of Cutaneous Microcirculation in the Foot Using Post-Occlusive Reactive Hyperemia

Running title: Evaluation of microcirculation in the foot

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Abstract:

Objectives: Cutaneous microcirculatory impairments are associated with skin injury to the foot. Post Occlusive Reactive Hyperemia (PORH) is one of the quick and easy methods to assess microcirculatory function. However, there are variations in the protocols currently used. Hence, this study aimed to systematically investigate the reproducibility of PORH protocols with minimal occlusion time in the foot.

Methods: PORH was measured using 12 different protocols (3 occlusion times, 2 occlusion sites and with or without temperature control) in 25 healthy adults. Each of the 12 different protocols was tested 3 times and the Intraclass correlation coefficient (ICC) was calculated.

Results: ICC showed that that ankle level occlusion produced moderate to excellent reproducibility for most PORH measures. In the right foot, 30- and 60- seconds ankle level occlusion without temperature control showed ICC of >0.40 for all parameters except the area of hyperemia (ICC = -0.36) and biological zero to peak flow percent change (ICC = -0.46). In the left foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.40 for all parameters except time to latency (ICC = 0.29), after hyperemia (ICC = 0.37), and max (ICC = -0.01), and area of hyperemia (ICC =-0.36). But the 60- seconds protocol showed ICC >0.40 for all except time to max (ICC = 0.38). In the hallux protocols, all three 10-, 30- and 60-seconds protocols without temperature control showed moderate to excellent reproducibility (ICC >0.40). In most cases, the temporal and area under the perfusion-time curve parameters showed poor reproducibility.

Conclusion: PORH can be tested efficiently with a minimal occlusion time of 10-seconds with hallux occlusion and 30-seconds with ankle occlusion in the foot. This can suggest that microcirculatory assessment is feasible in routine practice and can potentially be included for routine assessment of foot in people with diabetes.

Keywords: Microcirculation, PORH, Reactive Hyperemia, diabetic foot, plantar soft tissue

List of abbreviations:

Post-Occlusive Reactive Hyperemia – PORH

Intraclass Correlation Coefficient - ICC

Ankle Brachial Index - ABI

Toe Brachial Index - TBI

Endothelium-Derived Hyperpolarizing Factors - EDHFs

Laser Contrast Speckle Imager - LSCI

Peripheral Arterial Obstructive Disease - PAOD

Laser Doppler Perfusion Monitoring – LDPM

Perfusion Units - PU

Rest Flow - RF

Biological Zero - BZ

Peak Flow – PF

Time to Latency - TL

Time to Recovery – TR

Time to Half Before Hyperemia - TH1

Time to Max – TM

Time to Half After Hyperemia - TH2

Area of Occlusion - AO

Area of Hyperemia - AH

Area of Hyperemia/Area of Occlusion; Hyperemia repayment ratio - AH/AO

Background:

For long the peripheral vascular function assessments have relied on macrocirculatory measures such as Ankle Brachial Index (ABI) and Toe Brachial Index (TBI). Even assessment of peripheral vascular function in at-risk individuals for complications such as foot ulcers, clinical decisions, and risk stratification are based on guidelines that have been traditionally limited to measures such as ABI and TBI [1–5]. However, in recent times the measurement of microcirculation has gained importance, especially in the field of diabetes. Previous research shows that microcirculation plays a major role in diabetic foot-related complications [6–8]. The recent development in medical technologies has facilitated the non-invasive assessment of the microcirculation. Various provocation tests are used to assess the cutaneous microcirculatory responses such as heat provocation, cold provocation, postural changes and application of pressure stimuli [9–11]. One such test is Post Occlusive Reactive Hyperemia (PORH), which is the transient increase in blood flow in the organ or tissue that occurs following a brief period of arterial occlusion [12,13]. The current study's literature review focused on identifying the knowledge gaps in measuring PORH at the foot and the aims and objectives of this research were to address some of the key gaps to strengthen the existing evidence base in order to suggest its future application in diabetic foot syndrome.

PORH is known to be primarily an endothelial-dependent process, however it involves both endothelial-dependent and independent mechanisms [14,15]. The hyperemic response is a result of the shear stress, the tangential frictional force acting at the endothelial cell surface caused by arterial occlusion. The endothelium releases vasodilatory substances in response to the mechanical stimulus [14]. Various factors are known to contribute to the vasodilation which are myogenic, neurogenic, humoral and other local factors such as potassium ions, hydrogen ions, carbon dioxide, catecholamines, prostaglandins, and adenosine [14,15]. Endothelial nitric oxide and other endothelium-derived agents, such as prostaglandins and endothelium-derived hyperpolarizing factors are particularly known to play a role in the mechanism of PORH. Apart from these substances, the sensory nerves contribute to the PORH mechanism [15–20]. PORH is a quick, easy and useful method to assess microcirculation in the foot. However, the methods

and equipment used to measure PORH vary widely. Also, there were three distinctive variations exist in the protocols: occlusion time, use of temperature control at the probe site and occlusion site.

The review of existing literature identified a range of equipment that has been used to measure PORH. Recently, there are various types of equipment available that measure the change in blood perfusion proficiently such as commonly used Laser Doppler flowmetry or fluxmetry system with a pressure unit [15,21] or Laser Speckle Contrast Imager (LSCI) which helps to visualize the reactive hyperemia in real-time [22]. The Laser Doppler flowmetry or fluxmetry system have exclusive software programs that facilitate to run automated protocols and monitor various measures of interest continually. So far, the evidence on the reliability of measuring PORH is scarce [23]. The study by Barwick et al. (2015) compared the use of various techniques but no attempt was made to compare the protocols. Therefore, there is a need for more studies to enable the reliable and practical measurement of PORH in a clinical setting [23].

As highlighted earlier, the protocols used to measure PORH varies widely and there is no standard protocol. Firstly, in terms of occlusion time, studies have used occlusion times ranging from 30- to 180-seconds in people with peripheral arterial disease, diabetic foot ulcers and history of ulcers [15,20–22]. Whilst these studies pave a way to understand the importance of measuring PORH and its relevance to various complications, there remains a need for more evidence to support the selection of protocols in order to systematically investigate PORH in minimal time. Morales et al. (2005) recommended a protocol to measure PORH in people with peripheral arterial obstructive disease [21]. According to that study conducted in people without diabetes, the PORH measurements were obtained with an occlusion that lasted for a maximum of 3 minutes or 1 minute in case of strong leg pain and the entire (both acclimatization and measurement) session lasted for 33 minutes without stops [21]. But, these long provocation tests in people with diabetes might trigger tissue damage, discomfort and pain, especially when they present with various complications. In such conditions, tests that can be conducted in a minimal time may be useful in order to decrease the risk of tissue damage, discomfort and pain.

Secondly, in terms of using temperature control at the probe site, the recommendations vary. Some studies recommend temperature control at the probe site and in contrast others do not recommend the use of it [15,21,23]. However it needs to be noted that the study population varied in these studies, especially

in terms of including people with diabetes [15,21,23]. Temperature is known to influence microcirculation, hence an optimal temperature of 32-33℃ is recommended in order to standardize the methods for cutaneous vascular evaluation. However, this may not help to identify other physiological differences [21,23–25]. A previous study has demonstrated the relationship between PORH measure (time to peak) and sensory neuropathy, which is the involvement of small sensory nerve fibers [20]. This suggests the role of sensory nerves in mediating the cutaneous microcirculation. The foot temperature in people with diabetes varies incredibly. People with diabetes may either present with warm or cold feet depending on the presence of neuropathy and its type, based on which the protocols for PORH needs to be customized [26]. Therefore, the reproducibility of protocols with and without temperature control needs to be evaluated.

Thirdly, different studies have used different occlusion sites [15,21,23]. Thigh occlusion may be better whilst assessing PORH in people with complications such as arteriosclerosis, diabetes, renal insufficiency and such as it may be more reliable than ankle [21,27]. However, people with diabetes tend to be overweight/obese and often present with various complications (predominantly vascular and neuropathic). Hence, it may not be convenient and always possible to perform thigh occlusion. ABI is a common macrovascular measure in people with diabetes. TBI is measured in people with arterial calcification where ABI could be unreliable [28,29]. Considering how these measures of macrocirculation are measured, similar strategies can be applied whilst measuring PORH by occluding the blood flow at either the ankle or hallux site. Furthermore, in people with digital amputations hallux occlusion is impossible. This necessitates the evaluation and comparison of reproducibility of PORH protocols measured with occlusion at the ankle and hallux sites.

In summary, considering the three key gaps in the literature, this study aimed at investigating reproducibility of PORH protocols in microvascular assessment of foot in healthy young adults. The objective was to investigate a combination of occlusion time, occlusion site and the skin temperature control protocols that can be reproducible.

Methodology:

Participants

There were 25 healthy participants (15 females and 10 males) who participated in this study, which was conducted upon obtaining University Ethics Committee's approval. The participants were recruited through convenience sampling. Any adult over the 18 years with no severe neurological or vascular issues and no major vascular trauma or injury (bleeding, bruising, hematoma and fractured bones) that affects circulatory measurements could participate in the study. The participants were requested not to consume any caffeinated or alcoholic beverages 2 hours before the study as this is known to affect vascular measures [30–32]. Besides, the participants were requested not to engage in any strenuous exercise of any form 2 hours prior to the study [33]. The participants upon arrival to the Biomechanics laboratory were familiarized to the settings and the protocol. The test was performed by a single observer with a medical and clinical research background. .

Protocol

The participant was requested to lie supine on the couch. The nature of the tests required the participants to be as still as possible during the recording as even minor movements cause artefacts. The study commenced after a minimum of 15 minutes of acclimatization. The laser Doppler probes (contact area 1 cm^2 each) were secured using a double-sided adhesive tape from on the distal/plantar aspect of the hallux for ankle and toe pressure and PORH measurements or the pulp of the index finger for the arm pressure measurements. The ABI and TBI were measured as they are common macrocirculatory measures to confirm healthy peripheral vascular status of participants. The PORH occlusion was at the ankle and hallux levels (one followed by other), while the cuffs were inflated to a supra systolic pressure (~200 mmHg). Firstly, the PORH protocols included the use of three different occlusion times 10, 30 and 60 seconds. The recording for 60 seconds protocol consisted of two minutes baseline, occlusion for 60 seconds and two minutes of post-occlusion. The recording for 30 seconds protocol consisted of one-minute baseline, occlusion for 30 seconds and one minute of post-occlusion. The recording for 10 seconds protocol consisted of 30 seconds baseline, occlusion for 10 seconds and 30 seconds of post-

occlusion. Secondly, the same protocols were tested under two different conditions without and with temperature control of 33℃ at the probe site. Finally, the same set of protocols were tested with occlusion at two different sites, ankle and hallux. Both right and left feet were evaluated simultaneously. On the whole, there were 12 protocols (Figure 1) tested over 2.5 to 3 hours in a sequential manner and the rest between each testing was 60 seconds.

Equipment

The laser Doppler flowmetry (Periflux system 5000 manufactured by Perimed, Stockholm, Sweden) system was used for data collection. There can be several main systems, each equipped with four functional units to have the desired number of channels that facilitates simultaneous measurements of various vascular parameters. The system was a single main unit with 4 different functional units including: 2 perfusion units, 1 temperature unit and 1 pressure unit. The pressure unit helps to simplify and standardize tests such as peripheral vascular pressure measurements and PORH. The thermostatic laser Doppler probes were used to allow temperature monitoring for protocols without temperature control and to precisely maintain a temperature of 33℃ for protocols with temperature control at the measurement site. The kit with two Laser Doppler Perfusion Monitoring (LDPM) units allowed simultaneous blood pressure measurements at both arms followed by both ankles and halluces. The system was connected to a laptop with exclusive software, PeriSoft that helps to run the protocols in a sequential manner and record data. The steps for the protocols and the sequence of events were defined in order to facilitate effective, precise and replicable data collection across participants. The inflation of the pressure cuffs was manual; however, the deflation was automatic. The data for ABI, TBI and the PORH measures were collected using the same equipment.

Data analysis

For every participant, each of the 12 protocols, three trials were performed. Upon completion of each test, the PORH area, which included a baseline, occlusion trough and a hyperemic area was marked (Figure 2) for the system to autogenerate the results with values for PORH measure. This was done for every trial. Then, the reproducibility for the 14 parameters in Table 1: Rest Flow (RF), Biological Zero

(BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO) was analyzed through SPSS and Microsoft EXCEL was used for collating information. For every protocol, for each of these 14 measures, ICC between the three trials was statistically analyzed. Regarding ICC values, >0.75, 0.50-0.75, <0.50 were considered to be excellent, moderate (fair-to-good) and poor reproducibility [23,34,35]. All these statistical tests were conducted for right and left foot data separately.

Results:

The mean (standard deviation) age of the participants was 26.9 (9.2). The average height, weight and BMI were 168.1cm (14.2) and 72.9 kg (16), and 26.3.kg/m^2 (5.6), respectively. There were 14 different PORH measures recorded by the equipment (Figure 2). The mean with error bars (95% Confidence intervals) for 3 different measures of PORH across 12 protocols is provided in Figures 3 and 4. For the right and left foot, the mean and the trend across protocols for temporal parameters TM and TR are presented in Figures 5 and 6 and Figures 7 and 8, respectively. The ICC values, along with mean and standard deviation for key parameters of interest are presented in Table 2.

Overall, ICC showed moderate to excellent reproducibility for most PORH measures with ankle level occlusion. The perfusion parameters (RF, BZ and PF) showed excellent reproducibility with all protocols. Closer inspection of the results showed that the 30- and 60-seconds without temperature control protocols showed moderate to excellent reproducibility for most PORH measures. In the right foot, 30-seconds ankle level occlusion without temperature control showed ICC of >0.50 for all parameters except AH (ICC = -0.36) and AH/AO (ICC = 0.48). More specifically, the ICC Values were > 0.75 for RF, BZ, PF, RF-BZ, BZ-PF, TL, TH, TH2 and AO parameters (p value <0.05). The 60-seconds ankle level occlusion without temperature control in the right foot showed ICC of >0.50 for all parameters except BZ-PF percent change (ICC = -0.46). The ICC values were > 0.75 for RF, BZ, PF, RF-BZ, RF-PF, TL and TM (p value< 0.05).

Similarly, in the left foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.50 for all parameters except three temporal parameters (ICC values TL= 0.29, TH2 = 0.37, TM = -0.01) and AH (ICC =-0.36). With the 60- seconds protocol showed ICC >0.50 for all except TM (ICC = 0.38).

In the hallux protocols, all three 10-, 30- and 60-seconds protocols without temperature control fared well as they had a minimum of 11 out of 14 (78.57%) PORH measures that showed moderate to excellent reproducibility (ICC >0.50) (Table 2).

When temperature control was used at the probe site, be it with the ankle or hallux level occlusion, in most cases the temporal and area under the perfusion-time curve parameters showed poor reproducibility with ICC values <0.50.

In addition to the PORH measures, the ABI and TBI was measured. The mean ± standard deviation for ABI was 1 ± 0.13 and for TBI was 0.66 ± 0.14.

Discussion:

General remarks

The use of software aided protocols helps to pre-define the steps and abled to run tests systematically and time-effectively across participants. Each of the 12 protocols was performed at ease in minimal time. For instance, the 60 seconds protocol took only a maximum of 5 minutes and 10 seconds in total: 10 seconds preparation, 2 minutes baseline, 1-minute occlusion and 2 minutes hyperemia recording.

This study was conducted in healthy participants to assess the feasibility of conducting the test and reproducibility of various protocols. This study showed that the output for 30- and 60-seconds protocols produced more consistent results in the ankle as the output missed at least one parameter for a minimum of one of three trials for the 10 seconds protocol. A possible explanation for this might be that the 10 seconds occlusion time was insufficient to produce a hyperemic response. This is in line with the existing literature which suggests that the hyperemic response corresponds to the occlusion time [19]. The greater the occlusion time greater the hyperemic response; this is because longer the period of occlusion,

the greater the metabolic stimulus for vasodilation resulting in an increased in peak flow and prolonged duration of hyperemia [12,19]. Depending on the time taken to occlude the tissue blood supply, the reactive hyperemia increases four to seven times the baseline in the tissue and lasts from few seconds to hours in relation to the initial occlusion time. In this instance, the use of 10 seconds occlusion time at the ankle did not sufficiently provoke the vasodilation for a decent hyperemic response. However, this was not the case with hallux level occlusion. This may be due to the smaller surface area and the type of vessels in the hallux as compared to the ankle region.

Another key observation was that there was increased perfusion when protocols with temperature control were used, which suggests the role played by the thermoreceptors on the microcirculation and the effect of foot temperature on the cutaneous microcirculation. This can be observed in figure 3 and figure 4, where the perfusion measures RF, BZ and PF are higher in protocols with temperature control of 33°C compared to the corresponding values when temperature was not controlled. This is consistent with the suggestions from a previous study on the influence of temperature on cutaneous microcirculatory responses such as pressure-induced vasodilation [24,36]. Certain microcirculatory responses such as pressure-induced vasodilation are known to be absent even in healthy subjects due to low foot temperature and the role of mechanothermal receptors in such instances are highlighted [13,24,36]. These observations suggest an association between the small fiber nerve function and skin microcirculation. These findings generate interesting questions regarding the nature and extent of microcirculatory changes influenced by temperature variation. Further comparative analysis would be useful to understand the differences. While this was not within the scope of this study, it could be an area for further work.

Key remarks

The key measures of interest were the PF, RF to PF percent change, TM and TR as these measures are similar to the common parameters discussed in previous literature [15,21,34]. The PF parameter showed excellent reproducibility (ICC > 0.75; p-value < 0.05) across all 12 protocols. Similarly, RF to PF percent change showed either moderate or excellent reproducibility (ICC > 0.50; p-value < 0.05) across all 12 protocols, except with 10-seconds occlusion at the right and left ankles without temperature

control at probe site where it showed poor reproducibility (ICC = 0.47 and 0.15, respectively; p-value = 0.03 and 0.3, respectively). But, it is important to note that as highlighted earlier, 10-seconds protocols at the ankle never generated consistent reports for any parameters.

The ICC values for TM and TR showed variations across protocols (Table 2). Also, it can be observed from Figure 5 and Figure 6 that the TM was higher with ankle occlusion protocols than in the hallux occlusion ones in both feet. Furthermore, as seen in Figure 7 and Figure 8, the TR shows a similar trend. This implies an existing relationship between the occlusion site and the time taken for a hyperemic reaction. The reperfusion of the entire foot may have taken longer compared to the hallux. These findings help to realize the importance of understanding the use of different protocols and their influence on the PORH measures. Further comparative analysis may aid to understand these differences.

Previous research has indicated mixed results on the reliability of PORH in the upper limb [34,37,38]. As indicated by Barwick et al. (2015) there is a wide variation within the literature on the reliability of PORH. A possible reason being Laser Doppler Flowmetry system is extremely sensitive and the measurement varies depending on the probe location i.e. being directly over an arteriovenous anastomosis. Furthermore, PORH measures are sensitive to various factors such as temperature [23]. But, Barwick et al. (2015), found that PORH can be measured reliably, especially with the use of temperature control. Similarly, the current study found that PORH can be measured reliably. However, the current study found moderate to excellent reproducibility when using no temperature control in contrast to the findings from the previous study with hallux level occlusion [23]. A potential reason for this could be the fact that the current study was conducted in healthy participants whereas the other study was conducted in people at risk of peripheral arterial disease. As suggested in the literature, temperature control at the probe site may aid to minimize the variations in the perfusion measures as mere control for room temperature may not be adequate [23]. However, measurements obtained using temperature control at the probe site are criticized to be less physiologically relevant [21,23,25]. Microcirculatory measurements are influenced by the underlying pathological conditions, therefore, deeper understanding of variations with protocols may aid to obtain PORH measures that are methodologically sound and also physiologically relevant.

Future implications

The comparison of 12 different protocols in this study helped to assess the feasibility and reproducibility of results whilst systematically investigating PORH in the foot. The study is a stepping-stone to suggest incorporation of less-time consuming microvascular assessments in routine practice. According to these data, we can infer that the 30-and 60-seconds protocols produced consistent results. Thus, the present study raises the possibility that PORH can be measured using minimal time as little as 30 seconds in the foot. These protocols can be easily replicated in a clinical or research setting.

Future application in diabetic foot syndrome

In people with type 2 diabetes, it was found that certain PORH measures such as Time to peak and percent change from baseline were associated with the presence of peripheral sensory neuropathy, cardiac autonomic deficits, critical-ischaemia in the feet and previous history of ulcers/amputations [15,20,22]. These findings from previous research support the need for incorporating microcirculatory investigation in people at risk for diabetic foot complications. People with diabetes can present with a plethora of symptoms based on several foot complications, which makes it challenging to perform microvascular assessments. For example, ABI has been reported to be less reliable in people with arterial calcification and in such population, TBI is more reliable [28,29]. The results of the current study showed that PORH assessment was reproducible in both ankle and hallux occlusion, which can be useful when a decision must be made to select an occlusion level. This may have practical implications for determining the risk of diabetic foot complications, especially with ulcer prediction, wound healing and prevention of amputation.

Strengths and Limitations

This study tested the same session reproducibility. An advantage of this same session testing (similar to a real-world setting) is that the factors that influence variations in physiological measures are restricted. In contrast, the limitation is that the day to day variations in reproducibility and the potential factors influencing the measures were not studied. The test was performed by a single observer. The study examined reproducibility in healthy subjects. Although the findings from the study hold a value

from a methodological standpoint, its application to detect dysfunction in people with pathological conditions needs to be determined. As mentioned earlier, PORH assessment using Laser Doppler Flowmetry system is relatively quick and easy to perform in comparison to thermal provocation tests. However, the equipment is expensive. For this reason, it is used more for research purposes rather than clinical use at present. If the assessment aids with early diagnosis and prevention of adverse complications such as ulcers and amputations, it may prove to be beneficial in a long-term. This needs further investigation. Additionally, there is scope to investigate the cost-effective options for microcirculatory assessments for clinical use.

Conclusion:

This study assessed the reproducibility PORH parameters measured using 12 different combinations of PORH protocols in young healthy adults in the foot. The key highlights are that PORH can be reliably tested and in a very little time. The 10-seconds occlusion time was sufficient to induce a hyperemic response with occlusion at the hallux but not the ankle, which did affect the reproducibility. The protocol using 30- and 60- seconds occlusion time fared well in comparison to 10-seconds protocol and can be considered the most minimal time for ankle occlusion.

Data availability:

All original anonymized data for the study is available.

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Table 1: Various PORH Measures

The table shows various measures of PORH in the report generated by Laser Doppler Flowmetry/Fluxmetry system

Perfusion measures (PU)	**Percentage change measures (%)**	**Time measures (milliseconds)**	**The area under the curve**
RF - Rest flow **Baseline blood perfusion**	RF - BZ: Percent change	TL - Time to latency Time taken to reach baseline flow	AO - Occlusion area (Unit*sec.) The area under the occlusion curve
BZ - Biological zero **Temporary cessation of the blood flow during occlusion**	BZ - PF: Percent change	TR - Time to recovery Time taken to recover baseline level after the occlusion is released	AH - Hyperemia Area (Unit*sec.) The area under the hyperemic curve
PF - Peak flow **Maximum perfusion after the release of occlusion**	RF - PF: Percent change	TH1 - Time to half before hyperemia Time taken after the release of the occlusion for perfusion to reach the midpoint between no-flow and peak flow	AH/AO - Hyperemia repayment (ratio)
		TM - Time to max Time taken after the release of the occlusion for perfusion to reach peak flow	

		TH2 - Time to half after hyperemia Time taken after the occlusion release for perfusion to reach the midpoint between peak flow and baseline	

Table 2: ICC for PORH parameters in the Foot

Right Foot				Left Foot			
PF				PF			
	Protocols	ICC	SIG		Protocols	ICC	SIG
ANKLE	30-seconds OT without TC	0.96	0.00	ANKLE	30-seconds OT without TC	0.96	0.00
	30-seconds OT with TC	0.98	0.00		30-seconds OT with TC	0.96	0.00
	60-seconds OT without TC	0.98	0.00		60-seconds OT without TC	0.98	0.00
	60-seconds OT with TC	0.96	0.00		60-seconds OT with TC	0.92	0.00
HALLUX	10-seconds OT without TC	0.99	0.00	HALLUX	10-seconds OT without TC	0.97	0.00
	10-seconds OT with TC	0.97	0.00		10-seconds OT with TC	0.93	0.00
	30-seconds OT without TC	0.97	0.00		30-seconds OT without TC	0.99	0.00
	30-seconds OT with TC	0.97	0.00		30-seconds OT with TC	0.97	0.00
	60-seconds OT without TC	0.98	0.00		60-seconds OT without TC	0.98	0.00
	60-seconds OT with TC	0.95	0.00		60-seconds OT with TC	0.95	0.00
RF-PF Percent Change				RF-PF Percent Change			
	Protocols	ICC	SIG		Protocols	ICC	SIG
ANKLE	30-seconds OT without TC	0.65	0.00	ANKLE	30-seconds OT without TC	0.85	0.00
	30-seconds OT with TC	0.73	0.00		30-seconds OT with TC	0.54	0.01
	60-seconds OT without TC	0.92	0.00		60-seconds OT without TC	0.88	0.00
	60-seconds OT with TC	0.61	0.00		60-seconds OT with TC	0.52	0.01
HALLUX	10-seconds OT without TC	0.90	0.00	HALLUX	10-seconds OT without TC	0.94	0.00
	10-seconds OT with TC	0.95	0.00		10-seconds OT with TC	0.89	0.00
	30-seconds OT without TC	0.94	0.00		30-seconds OT without TC	0.94	0.00
	30-seconds OT with TC	0.87	0.00		30-seconds OT with TC	0.86	0.00
	60-seconds OT without TC	0.89	0.00		60-seconds OT without TC	0.93	0.00
	60-seconds OT with TC	0.63	0.00		60-seconds OT with TC	0.64	0.00
TM				TM			
	Protocols	ICC	SIG		Protocols	ICC	SIG
ANKLE	30-seconds OT without TC	0.58	0.01	ANKLE	30-seconds OT without TC	-0.01	0.49
	30-seconds OT with TC	-0.28	0.73		30-seconds OT with TC	0.33	0.12

	60-seconds OT without TC	0.76	0.00		60-seconds OT without TC	0.38	0.08
	60-seconds OT with TC	0.45	0.04		60-seconds OT with TC	0.31	0.15
HALLUX	10-seconds OT without TC	0.83	0.00	HALLUX	10-seconds OT without TC	0.72	0.00
	10-seconds OT with TC	0.21	0.24		10-seconds OT with TC	0.47	0.03
	30-seconds OT without TC	0.71	0.00		30-seconds OT without TC	0.75	0.00
	30-seconds OT with TC	0.60	0.00		30-seconds OT with TC	0.77	0.00
	60-seconds OT without TC	0.52	0.02		60-seconds OT without TC	0.02	0.47
	60-seconds OT with TC	0.40	0.08		60-seconds OT with TC	0.31	0.09
TR				TR			
	Protocols	ICC	SIG		Protocols	ICC	SIG
ANKLE	30-seconds OT without TC	0.63	0.00	ANKLE	30-seconds OT without TC	0.60	0.00
	30-seconds OT with TC	-0.09	0.58		30-seconds OT with TC	0.35	0.11
	60-seconds OT without TC	0.67	0.00		60-seconds OT without TC	0.50	0.02
	60-seconds OT with TC	-0.13	0.61		60-seconds OT with TC	-0.24	0.70
HALLUX	10-seconds OT without TC	0.98	0.00	HALLUX	10-seconds OT without TC	0.90	0.00
	10-seconds OT with TC	0.94	0.00		10-seconds OT with TC	0.98	0.00
	30-seconds OT without TC	0.95	0.00		30-seconds OT without TC	0.96	0.00
	30-seconds OT with TC	0.82	0.00		30-seconds OT with TC	0.60	0.00
	60-seconds OT without TC	0.91	0.00		60-seconds OT without TC	0.77	0.00
	60-seconds OT with TC	0.77	0.00		60-seconds OT with TC	0.93	0.00
OT = Occlusion Time; TC = Temperature Control at probe site							
	>0.75 Excellent reproducibility			0.50 – 0.75 Moderate reproducibility			<0.50 Poor reproducibility

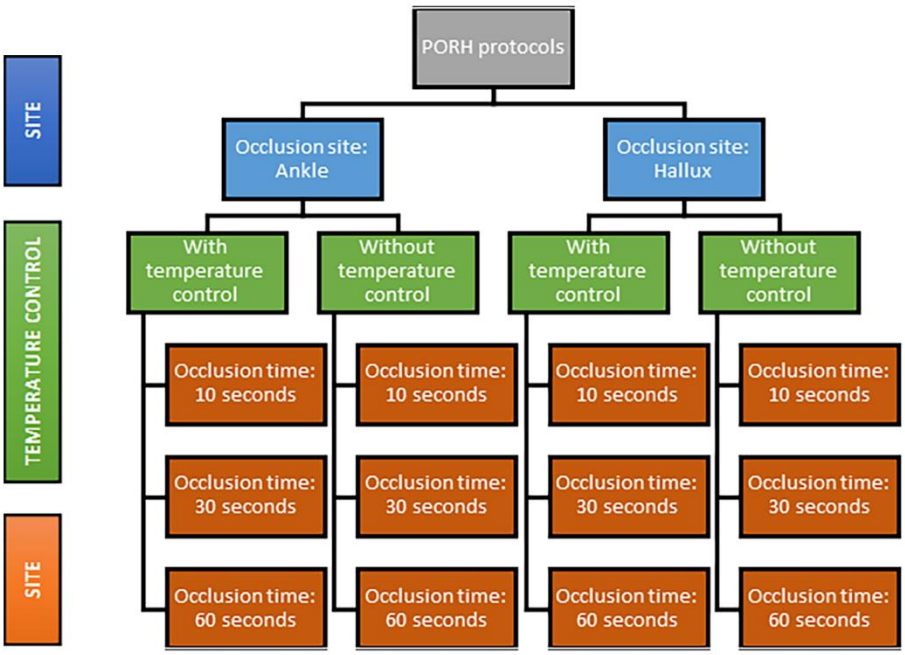

Figure 1: Protocols used in the current study

The image shows the 12 different protocols used in the current study based on 3 different occlusion times, 2 different occlusion sites and use of temperature control

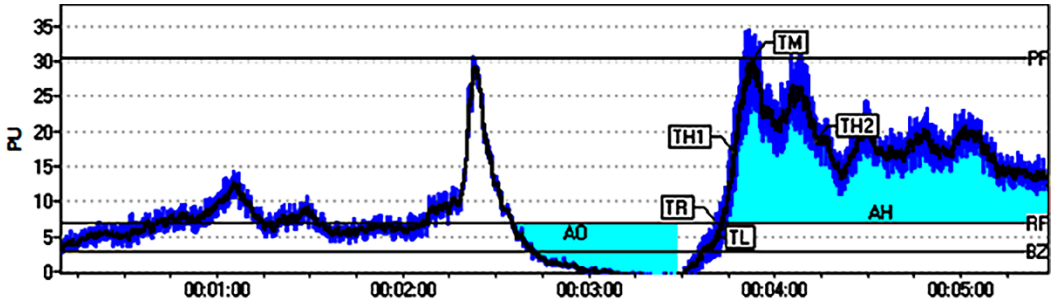

Figure 2: PORH graph showing various measures

Rest Flow (RF), Biological Zero (BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO)

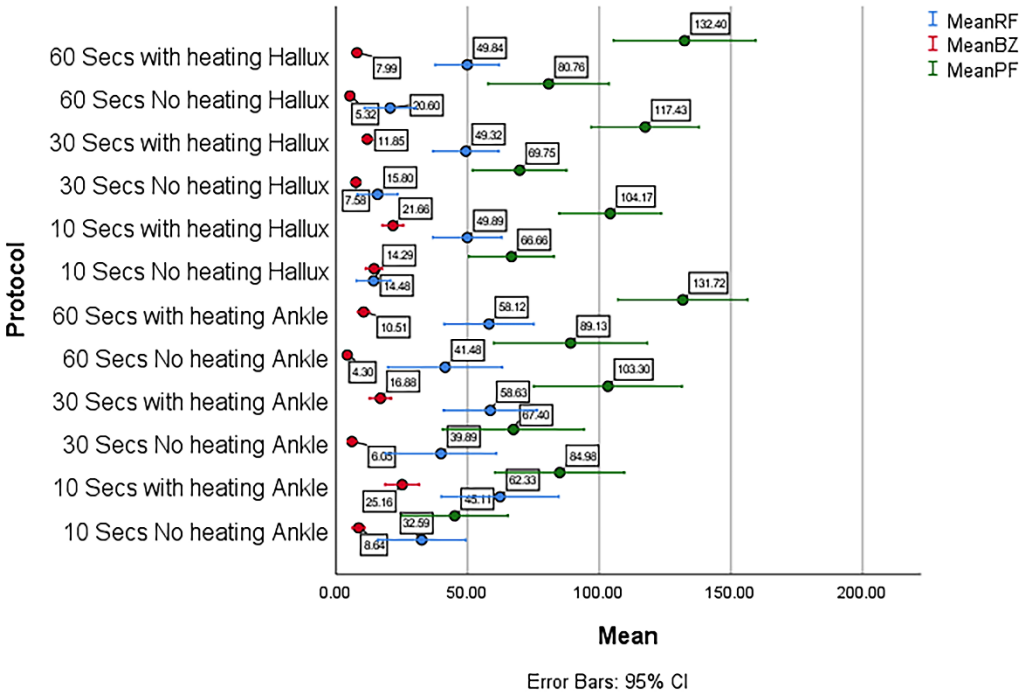

Figure 3: Right Foot: Mean of perfusion measures RF, BZ and PF (PU) across 12 protocols

Secs = Seconds; TC = Temperature Control

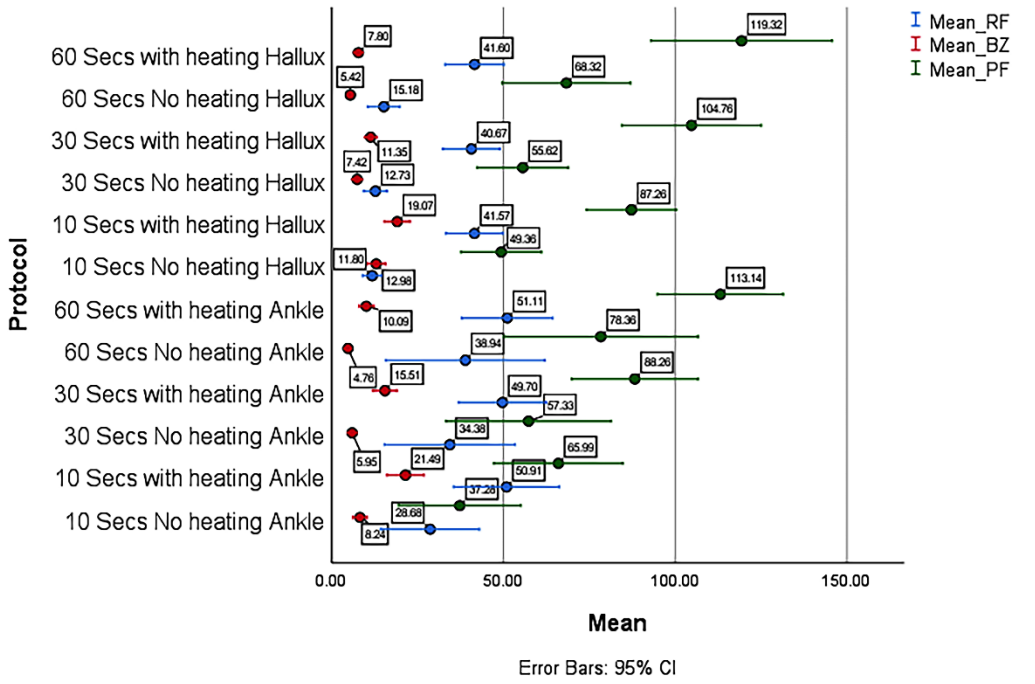

Figure 4: Left foot: Mean of perfusion measures RF, BZ and PF (PU) across 12 protocols

Secs = Seconds; TC = Temperature Control

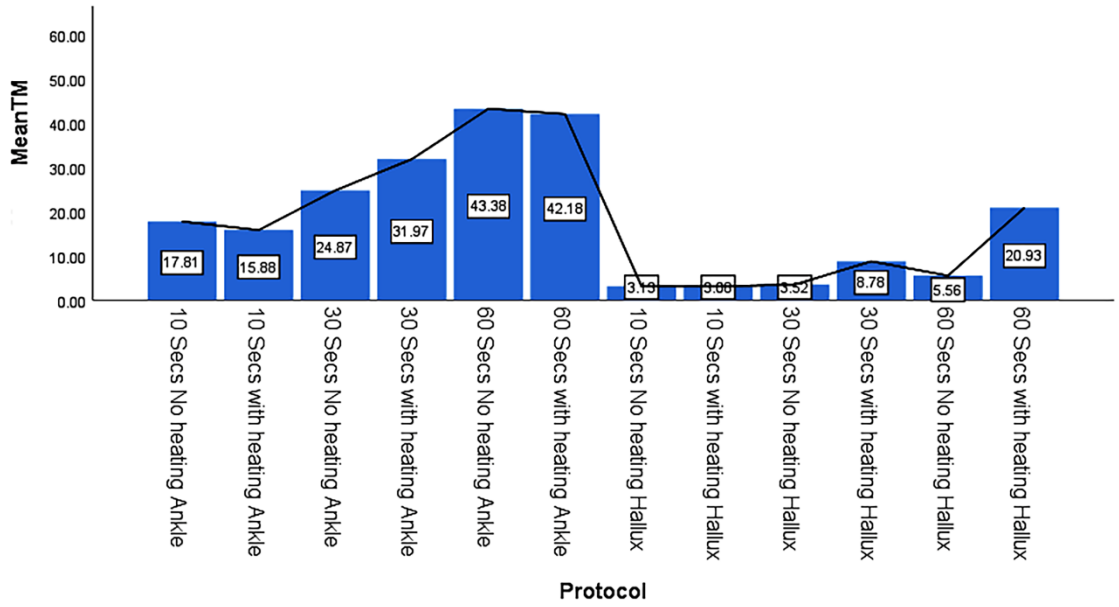

Figure 5: Right foot: Mean Time to max (seconds) categorized based on 12 protocols

Secs = Seconds; TC = Temperature Control

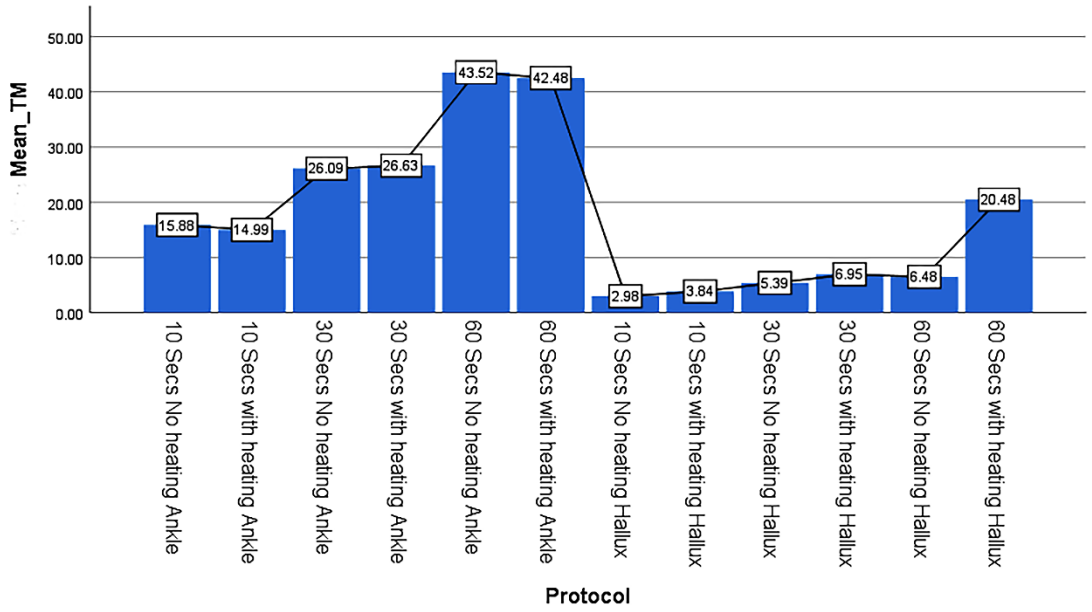

Figure 6: Left foot: Mean Time to max (seconds) categorized based on 12 protocols

Secs = Seconds; TC = Temperature Control

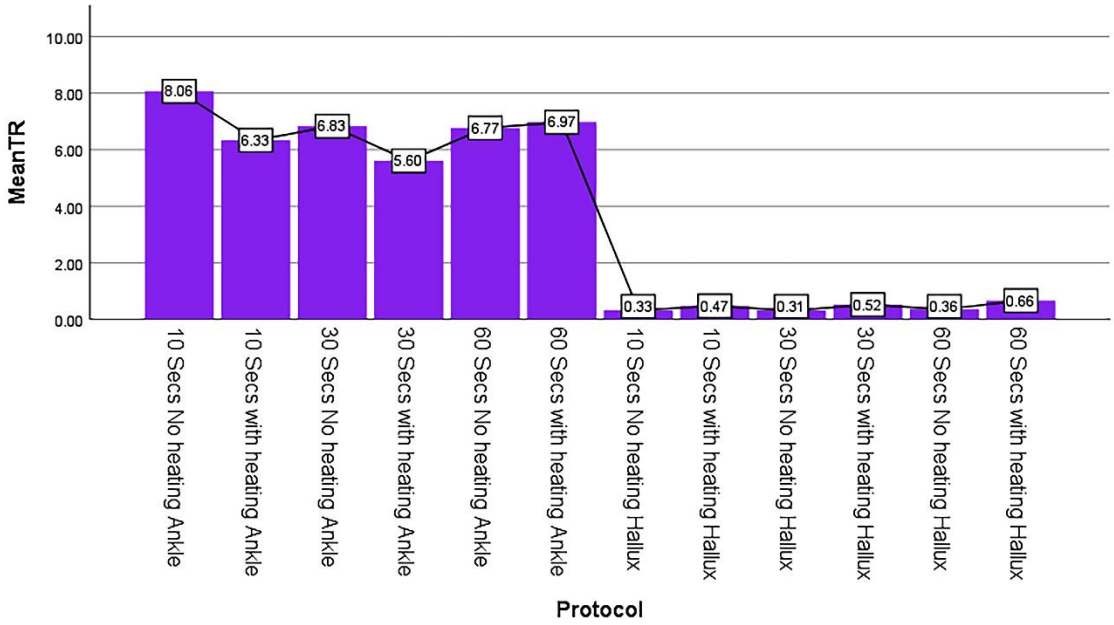

Figure 7: Right foot: Mean Time to Recovery (seconds) categorized based on 12 protocols

Secs = Seconds; TC = Temperature Control

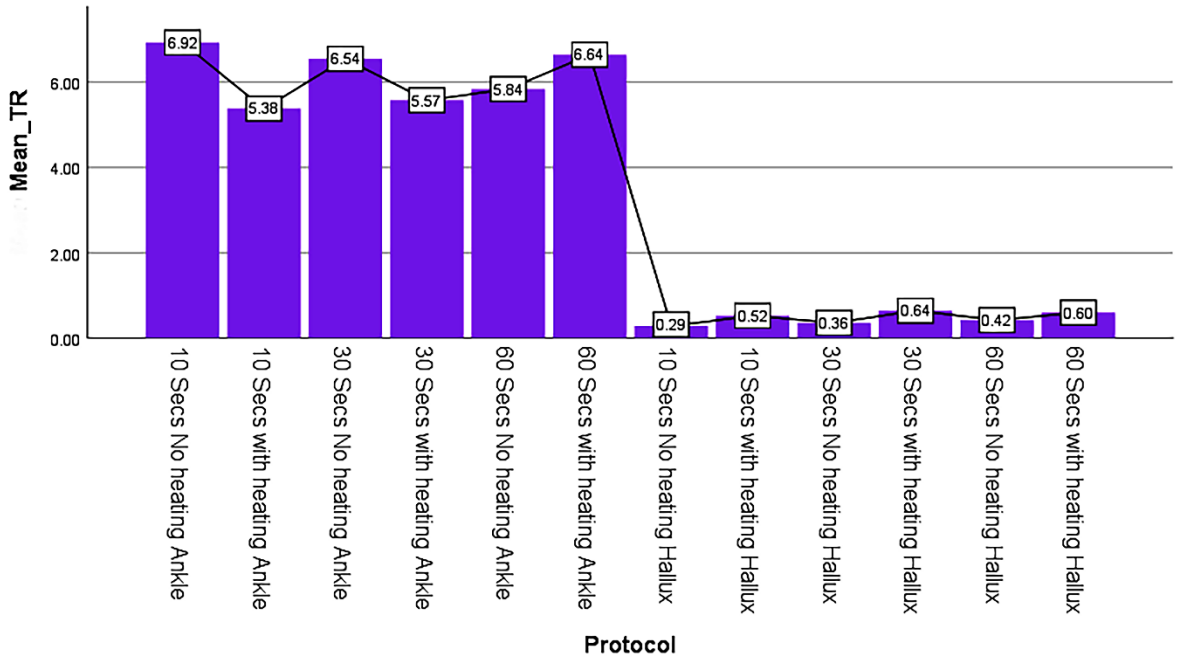

Figure 8: Left foot: Mean Time to Recovery (seconds) categorized based on 12 protocols

Secs = Seconds; TC = Temperature Control