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### Article

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# Reproducibility of Four Frequently Used Local Heating Protocols to Assess Cutaneous Microvascular Function

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## ABSTRACT

**Background.** Skin microvascular responses to local heating are frequently used to assess microvascular function. Several local heating protocols have been developed, all varying slightly in execution. The aim of this study was to determine the inter-day reproducibility of the four most commonly used local heating protocols in healthy young subjects.

**Methods.** Fifteen, healthy males ( $28\pm 5$  yrs, BMI  $25\pm 2$  kg/m<sup>2</sup>) attended two experimental trials 2-7 days apart. During each trial, baseline and maximal thermally stimulated forearm skin responses were examined simultaneously at four sites on the dominant forearm using laser Doppler flowmetry (LDF). The following heating protocols were adopted: 1. *Rapid 39°C* (0.5°C/5s), 2. *Rapid 42°C* (0.5°C/5s) 3. *Gradual 42°C* (0.5°C/2min30s) and 4. *Slow 42°C* (0.5°C/5min). The coefficient of variation (CV) was calculated for absolute flux, cutaneous vascular conductance (CVC; flux/mean arterial pressure, MAP) and CVC expressed as a percentage of maximal CVC at 44°C (%CVC<sub>max</sub>) at three different time points; baseline (33°C), plateau (39/42°C) and maximal (44°C).

**Results.** Reproducibility of baseline flux, CVC and %CVC<sub>max</sub> was 17-29% across all protocols. During the plateau, *Rapid*, *Gradual* and *Slow 42°C* demonstrated a reproducibility of 13-18% for flux and CVC and 5-11% for %CVC<sub>max</sub>. However, *Rapid 39°C* demonstrated a lower reproducibility for flux, CVC and %CVC<sub>max</sub> (all 21%). Reproducibility at 44°C was 12-15% for flux and CVC across all protocols.

**Conclusion.** This is the first study examining inter-day reproducibility across four local heating protocols. The good-to-moderate reproducibility of the *Rapid*, *Gradual* and *Slow 42°C* protocols support their (simultaneous) use to assess microvascular function. Using *Rapid 39°C* may require a greater number of subjects to detect differences within subjects.

**Keywords:** skin microcirculation, microvasculature, local heating, endothelial function, nitric oxide.

## INTRODUCTION

Microvascular dysfunction may predict the manifestation of future cardiovascular disease, preceding abnormalities in larger conduit arteries and arterioles [1-6]. The skin provides an easily accessible site to assess microvascular integrity through non-invasive methods, which can be used as an index of overall systemic vascular function. Control of the cutaneous microcirculation involves both neural and non-neural pathways [7]. Neurogenic reflexes and local chemical mediators, such as nitric oxide (NO), contribute towards the vasodilatory effect mediated by the vascular endothelium during local skin heating [8, 9]. Protocols that locally heat the skin are increasingly used in conjunction with laser Doppler flowmetry (LDF) to evaluate skin blood flow responses and microvascular function, particularly for comparing between healthy and diseased individuals and/or assessing responses to interventions.

There are currently several local heating protocols that are widely used to assess cutaneous microvascular function [8, 10, 11]. These protocols all aim to increase skin blood flow to maximal/near-maximal levels (39-42°C), but they vary in the rate at which the skin is heated (0.5°C per 5s, 2min30s or 5min) and/or the plateau at which the temperature is set (39°C vs 42°C) [8, 10-14]. Due to the differences in the plateau and the rate of skin heating, a different contribution of the vasodilator pathways to the local heating response is present. Rapid local heating (0.5°C per 5s) induces a transient axon-reflex (~5-10 min), produced via activation of heat sensitive sensory nerves and adrenergic nerves, followed by a more gradual, sustained vasodilatory response (20-30 min) that is partly (60-70%) NO-mediated [10]. A modification of this protocol, by maintaining the plateau phase at 39°C, is believed to lead to a larger contribution of

NO to the plateau phase [11]. Alternatively, gradually heating the skin (0.5°C per 2min30s or 5min) evokes a largely NO-mediated vasodilatory response, without producing an axon-reflex [8, 12].

Previous work found moderate to good inter-day reproducibility for all local heating protocols [12, 15-18], especially when data were expressed relative to maximal values [12, 15, 18]. However, no previous study examined the reproducibility of these local heating protocols within the same subjects and/or simultaneously. This latter aspect is of special importance, since simultaneous assessment of distinct heating protocols may achieve better insight due to the distinct dilator pathways involved. Therefore, the aim of this study was to simultaneously determine the inter-day reproducibility of four commonly used local heating protocols for assessing cutaneous microvascular function. We expect comparable reproducibility of all four protocols, which would facilitate simultaneous use of multiple local heating protocols within the same study.

## **METHODS**

### **Participants**

Fifteen healthy, male participants were recruited through local advertisement. All participants were healthy and non-smokers (28±5yrs, height 1.79±0.10m, weight 78.3±8.5kg, BMI 25±2kg/m<sup>2</sup>, mean arterial pressure (MAP) 79±5mmHg). Individuals with a medical history of hypercholesterolaemia (total cholesterol >6.5mmol/l), [19] cardiovascular disease and/or hypertension (systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg) [20, 21] were excluded. Participants were not taking any vasoactive medications or supplements. After being fully informed of the

methods, written informed consent was obtained from all participants. The study conformed to the Declaration of Helsinki and was approved by the Research Ethics Committee of Liverpool John Moores University.

## **Experimental Design**

All participants attended two experimental trials which were 2-7 days apart. During each trial, baseline and maximal thermally stimulated forearm cutaneous blood flow was examined simultaneously at four different sites on the dominant forearm using LDF. At each site, separated by ~5cm, a different local heating protocol was adopted: 1. *Rapid 39°C* [11], 2. *Rapid 42°C* [10], 3. *Gradual 42°C* [8], and 4. *Slow 42°C* [8]. The sites at the forearm were kept the same within subjects between the two testing days.

## **Experimental Measures**

All participants fasted for at least six hours and refrained from alcohol, food products high in polyphenols (dark chocolate, red wine), caffeine and exercise for 24h prior to testing [22]. Sips of water were permitted prior to testing to ensure that participants were well hydrated. All trials were conducted in a quiet, temperature controlled environment ( $23.0 \pm 0.4^\circ\text{C}$ ) [22, 23] and at the same time of day to reduce any circadian influences on vascular function [22, 24]. Stature (seca 217 stadiometer, seca UK, Birmingham, UK) and body mass (seca 767 calibrated electronic scales, Germany) were recorded using standardised protocols. Body mass index was calculated (BMI) as the body weight (kg) divided by the height squared ( $\text{m}^2$ ).

Following a 20-minute stabilisation period, the LDF equipment was calibrated using two generic points, 0 and 250 PU, a zeroing disk and motility standard, according to manufacturer's guidelines (Perimed AB, Järfälla, Stockholm, Sweden). Participants assumed a comfortable, supine position on a bed, with the head slightly elevated and the dominant arm relaxed, supinated and supported by a vacuum cushion to minimise microcirculatory fluctuations resulting from motion artefact [22, 23]. Four measurement sites on the volar aspect of the dominant forearm were randomly chosen  $\geq 2.5$ cm from the antecubital fossa and  $\geq 2.5$ cm from the distal radio-ulnar joint at the wrist, avoiding visible veins, hair follicles and dermatological lesions [23]. If necessary, the measurement sites were shaved 24h prior to testing to avoid any inflammatory response that may affect cutaneous blood flow. Following a 20-minute rest period, participants were instrumented for LDF measurements; four heating discs (Perimed 355, Perimed AB, Järfälla, Stockholm, Sweden) were placed  $\sim 5$ cm apart on the dominant forearm, with a 7-laser array probe (PF 413, Perimed AB, Järfälla, Stockholm, Sweden) placed into each heater and firmly attached to the skin using adhesive stickers. To ensure accuracy of measurement sites between trials, the relevant areas were marked on the skin following the first experimental trial. In addition, we took digital photographs and recorded measurements to the nearest millimetre using anatomical and skin-surface landmarks for reference.

Cutaneous blood flow was measured as a signal of red blood cell flux (RBCF) using the non-invasive technique of LDF (Periflux system 5000, Perimed AB, Järfälla, Stockholm, Sweden). The four local heating discs were connected to a heating unit (Peritemp 4005 heater, Perimed AB, Järfälla, Stockholm, Sweden) which was used to induce thermal hyperaemia and was manually controlled during the local heating

protocols. Baseline skin RBCF was recorded with the local heating disc temperature set at 33°C for 10-minutes for each measurement site. Subsequently, local skin temperature was heated according to four distinct protocols:

*Rapid 39°C* [11]. This recently introduced protocol (0.5°C per 5s, 30-min at 39°C, 20-min at 44°C) induces an axon-reflex and gradual plateau following local heating. By stopping the heating protocol at 39°C, the plateau phase is largely NO-mediated and causes dilation that is equivalent to 50% of the maximal response [11].

*Rapid 42°C* [10]. This classic local heating protocol (0.5°C per 5s, 30-min at 42°C, 20-min at 44°C) induces a rapid, transient axon-reflex which is followed by a more gradual, but sustained, heating response. The plateau phase represents 80-90% of the maximal response, and is partly (60-70%) NO-mediated [10, 25].

*Gradual 42°C* [8]. This adapted, shortened version of the *Slow 42°C* local heating protocol increases temperature to 42°C (0.5°C per 2-min 30s, 30-min at 42°C, 20-min at 44°C), and induces a slow heating response that is largely NO-mediated and reflects 80-90% of the maximal response [8].

*Slow 42°C* [8]. This validated, longer version of the former heating protocol induces a gradual, slow heating response (0.5°C per 5-min, 30-min at 42°C, 20-min at 44°C) that is largely NO-mediated, reflecting 80-90% of the maximal response.

*Haemodynamics.* Heart rate (HR) and blood pressure (BP) were recorded at the beginning and at the end of the 20-minute acclimation period using an automated sphygmomanometer (Dinamap V100, GE Healthcare, UK) positioned on the contralateral upper arm. Thereafter, MAP (mmHg) and HR (bpm) were recorded at 5-minute intervals throughout the local heating protocols. MAP was used to calculate



cutaneous vascular conductance (CVC; RBCF/MAP), thereby accounting for changes in skin blood flow resulting from variations in BP [10, 12, 23].

## **Data Analysis**

Cutaneous RBCF was indexed as perfusion units (PU) and represents local cutaneous blood flow, which was subsequently expressed as cutaneous vascular conductance (CVC), as described previously [23]. Artefact in the data, due to unwanted subject movement, was identified and removed prior to analysis. Baseline laser Doppler flux was averaged over a stable 10-minute period. The plateau phases during heating (39/42°C and 44°C) were averaged over the last 5-minutes of each protocol. Data at baseline and at the various plateau phases were also normalised to the maximal flux achieved at 44°C ( $\%CVC_{max} = [CVC / CVC_{max}] \times 100$ ). All data were collected in LabChart 7.0 (ADInstruments, Dunedin, New Zealand).

## **Statistical Methods**

Data were expressed as mean $\pm$ SD and statistical significance was set at  $P < 0.05$ . Mixed model RMANOVA was used to compare local heating protocols and paired Student's *t*-tests were used to examine day-to-day systematic bias of each local heating protocol. Bland-Altman plots were constructed to demonstrate individual variability. The coefficient of variation (CV) was calculated to assess the inter-day reproducibility of CVC and  $\%CVC_{max}$  at baseline, 39/42°C and 44°C. Prior to calculating the CV, a natural logarithmic transformation was applied to correct for heteroscedasticity of the data. For biological variables, a CV of <10% is considered good and <20% is acceptable [26]. A two-way ANOVA was used to examine BP within

each protocol and between test days (main effects of local temperature and visit). Data were stored and transformed within Microsoft Excel (Microsoft Office 2010, Microsoft Corporation), and statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

## RESULTS

Figure 1 shows representative skin blood flow traces for all local heating protocols, indicating the phases and associated local temperatures (33°C, 39/42°C and 44°C) that were used for analysis. There was a small but significant increase in MAP during the heating protocols ( $P=0.002$ ), consistent with a circadian variation. This gradual increase in MAP was not different between trials ( $P=0.39$ ) and no interaction effect was found between local temperature and trial ( $P=0.49$ ).

*Baseline.* Baseline cutaneous blood flow was not different between days when expressed as arbitrary flux or CVC (all  $P>0.05$ , Table 1). Also when expressed as %CVC<sub>max</sub>, cutaneous blood flow was not different between days for the heating sites for *Rapid 39°C*, *Rapid 42°C*, *Gradual 42°C* or *Slow 42°C* (Table 1). Inter-day variation of baseline cutaneous blood flow was 17.2-26.1% for flux, 17.4-24.8% for CVC and 18.6-29.2% for %CVC<sub>max</sub> (Table 1), with no differences between local heating sites.

*Local heating to plateau (39/42°C).* For all four protocols, inter-day cutaneous perfusion at the plateau phase was not different between days when expressed as arbitrary flux, CVC or %CVC<sub>max</sub> (all  $P>0.05$ , Table 2). Lower inter-day reproducibility

was found for the plateau phase of *Rapid 39°C* compared to *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* when data were expressed as flux, CVC or %CVC<sub>max</sub> (Table 2). When data were presented as %CVC<sub>max</sub>, CV was lower for *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* (Table 2). Bland-Altman plots demonstrated no obvious heteroscedasticity for the responses at the plateau phase (Figure 2).

*Maximal heating.* Perfusion, presented as flux of CVC, during maximal heating (44°C) was not different between days (Table 2). The maximum heating response resulted in a reproducibility of 12.3-15.0% when data were expressed as arbitrary flux and CVC (Table 2). This observation was valid across all four protocols. Bland-Altman plots demonstrated no obvious heteroscedasticity for the responses during maximal heating (Figure 3).

## **DISCUSSION**

The aim of this study was to explore the inter-day reproducibility of four commonly used (and simultaneously performed) local skin heating protocols for assessing cutaneous vascular function. Our findings suggest that inter-day variation of baseline cutaneous blood flow demonstrated poor-to-moderate reproducibility. Secondly, inter-day reproducibility of cutaneous blood flow responses to the plateau phase of the *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* protocols was moderate for flux and CVC, but good when data were presented after correcting for maximal perfusion (%CVC<sub>max</sub>). In contrast, inter-day reproducibility of perfusion during the plateau phase of the *Rapid 39°C* protocol was moderate-to-poor. Finally, maximal inter-day cutaneous blood flow demonstrated moderate reproducibility for arbitrary flux and CVC across all protocols,

which indicates that the maximum response was not affected by the preceding protocol of local heating. These observations have clinical impact for designing future studies, especially when multiple local heating protocols are being used simultaneously.

Protocols that locally heat the skin in combination with LDF are frequently used to assess microvascular integrity and index overall systemic vascular function [4]. Current protocols vary in methodology with fast [10, 11] or gradual [8, 12] rates of skin heating. Although studies have explored the reproducibility of individual heating protocols, the current study is the first to simultaneously assess reproducibility of multiple protocols. This is particularly important given that the simultaneous assessment of cutaneous vascular function using these distinct heating protocols may achieve better insight due to the distinct dilator pathways involved. Firstly, we found moderate-to-poor reproducibility for baseline perfusion, in agreement with our, and others, previous research [12, 18]. It is important to acknowledge that the reproducibility of baseline perfusion was independent of the site of measurement (i.e. the four different sites that underwent the distinct heating protocols). Similarly, maximal perfusion at 44 °C also demonstrated good agreement between the four measurement sites. This demonstrates that the reproducibility of the LDF technique to assess baseline or maximal perfusion is not dependent on the site of measurement, but also was not affected by the local heating protocol (e.g. rapid or gradual).

Although distinct heating protocols were used, we expected a comparable reproducibility for the plateau phase according to previous studies [12, 18]. In line with

our hypothesis, but also in agreement with previous studies [12, 18], moderate reproducibility was found for perfusion during the plateau phases of the *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* protocols. Again consistent with previous studies [12, 18], expressing data as a percentage of maximal perfusion resulted in an improvement of reproducibility. As CVC data is typically normalised to maximum to account for the heterogeneity of cutaneous vessel density, this may explain the increased reproducibility when data were normalised to %CVCmax. The use of integrated probes in the current and aforementioned studies, which allow an examination of a greater surface area of skin, may have contributed to the good-to-moderate reproducibility. Indeed, previous work on local heating using single point LDF probes report lower reproducibility [15, 17].

Despite the good agreement between the rapid, gradual and slow heating protocols to 42°C, poorer reproducibility was reported for forearm cutaneous vascular responses to the rapid heating protocol to 39°C. The poorer reproducibility of the *Rapid 39°C* protocol cannot be simply explained by the speed of increasing local temperature, especially since reproducibility of the *Rapid 42°C* and *Gradual 42°C* protocols were similar. The *Rapid 39°C* protocol was developed in order to isolate NO-dependent dilation and/or allow a better assessment of perturbations that may improve microvascular function due to the levels of skin blood flow achieved at this local skin temperature (~50% maximum) compared to those at 42°C (~90% maximum) [11]. Differences in reproducibility between the *Rapid 39°C* protocol and the other protocols may therefore relate to the differences in the level of skin blood flow achieved in the protocols. Skin blood flow was lower at the plateau phase of the *Rapid 39°C* protocol (i.e. ~50% of the maximum response) relative to the plateau phases of the *Rapid 42°C*,

*Gradual 42°C* and *Slow 42°C* protocols (80-90% of the maximum response). Such levels may provide more space for variation in perfusion, despite correcting the level of perfusion for differences in the maximum perfusion at 44 °C. One consequence of this observation is that a larger group size is required for the *Rapid 39°C* protocol compared to the other protocols to detect differences within subjects.

Using our data from the present study, we calculated the sample sizes required to show significant changes in %CVC<sub>max</sub> at 39/42 °C for within subject comparisons (e.g., pre and post interventions; Table 3). Assuming a power of 80% ( $\alpha=0.05$ ), the *Rapid 39°C* protocol would require 54 subjects to detect a 5.0% change in %CVC<sub>max</sub> at 39 °C compared to 27 subjects at 42 °C for the *Rapid 42°C* protocol. Again, assuming a power of 80% ( $\alpha=0.05$ ), the *Gradual 42°C* protocol would require 17 subjects to detect a 5.0% change in %CVC<sub>max</sub> at 42 °C and the *Slow 42°C* protocol would require 47 subjects. These sample size estimations demonstrate distinctly different requirements for studies that use both rapid and gradual local heating, possibly related to between protocol differences in underlying vasodilatory mechanisms.

The reproducibility of the *Rapid 42°C*, *Gradual* and *Slow 42°C* protocols were similar. Importantly, these local heating protocols induce skin vasodilation via different mechanisms. Whilst slowly heating the skin prevents significant axon-reflexes and leads to a largely NO-mediated response, rapidly heating the skin activates the axon-reflex and leads to a NO- and EDHF-mediated response [7]. The involvement of multiple vasoactive substances in the rapid heating response might have contributed to a larger variation in response to the *Rapid 42°C* local heating protocol. However,

similar coefficients of variation were evident for the CVC and %CVC<sub>max</sub> data in the *Rapid 42°C* and *Gradual 42°C* protocols. The specific underlying pathways leading to vasodilation in response to the distinct heating protocols may therefore provide useful synergistic insight into complementary vasodilatory mechanisms. Therefore, supported by the comparable reproducibility, we suggest that measuring multiple local heating protocols is easy, applicable and feasible, but may also provide better insight into the skin vasodilator mechanisms. **An alternative approach would be to use the same heating protocol across multiple sites to minimise any between-site differences. Averaging data across these sites would improve reliability and thereby reduce the sample size requirements. However, use of a single protocol is limited in only assessing one vasodilatory pathway, whereas a multi-protocol approach may elicit further mechanistic insight into vasodilatory responses observed.**

*Limitations.* We assessed inter-day reproducibility in a young, healthy population, thereby limiting extrapolation of our findings to individuals and patient groups with cardiovascular risk, such as older individuals, who commonly exhibit attenuated cutaneous blood flow responses to local heating [1, 8, 23, 27]. However, young and older subjects demonstrated comparable reproducibility of local heating in a previous study [18]. A further limitation is that females were excluded from this study to control for hormonal influences on vascular function [28-30] and, therefore, our findings are applicable to males only.

In conclusion, this is the first study to simultaneously examine the inter-day reproducibility of four local heating protocols, which are currently frequently used to assess cutaneous blood flow in humans. Our findings suggest that the reproducibility of baseline forearm skin perfusion assessment is poor to moderate, and is independent of the site of measurement. The *Rapid, Gradual and Slow 42°C* protocols exhibited superior reproducibility, particularly when data is expressed as %CVC<sub>max</sub>, compared to the *Rapid 39°C* protocol. Our data support the validity of repeated measures of cutaneous blood flow in response to local heating protocols, for use in epidemiological studies as an index of microvascular function. Furthermore, this data provides help in guiding future studies in calculating sample sizes necessary to detect differences, especially since differences are present between the different protocols.

## **CONFLICT OF INTEREST**

None of the authors have a conflict of interest.

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**Table 1.** Baseline cutaneous blood flow results. Data is presented as mean  $\pm$  SD. Inter-day reproducibility is presented as CV ( $\pm$  95% CI): light grey shading indicates CV: >21%; mid grey shading indicates CV: 10-20%.

	Trial 1	Trial 2	Between-day CV (%)	Paired t-test
<b>Baseline (33°C)</b>				
<i>Rapid 39°C</i>				
Absolute flux (PU)	21 $\pm$ 10	20 $\pm$ 9	26.1 (19.1 - 41.3)	0.57
Absolute CVC (PU/mmHg)	0.26 $\pm$ 0.12	0.25 $\pm$ 0.11	24.8 (18.2 - 39.2)	0.66
Maximal CVC (%CVC <sub>max</sub> )	9 $\pm$ 5	8 $\pm$ 4	29.2 (21.4 - 46.5)	0.51
<i>Rapid 42°C</i>				
Absolute flux (PU)	20 $\pm$ 9	19 $\pm$ 10	17.2 (12.6 - 26.8)	0.10
Absolute CVC (PU/mmHg)	0.26 $\pm$ 0.11	0.24 $\pm$ 0.13	17.4 (12.8 - 27.2)	0.26
Maximal CVC (%CVC <sub>max</sub> )	8 $\pm$ 4	8 $\pm$ 5	19.3 (14.2 - 30.3)	0.66
<i>Gradual 42°C</i>				
Absolute flux (PU)	19 $\pm$ 8	20 $\pm$ 12	19.9 (14.6 - 31.2)	0.93
Absolute CVC (PU/mmHg)	0.24 $\pm$ 0.10	0.25 $\pm$ 0.15	20.8 (15.3 - 32.7)	0.79
Maximal CVC (%CVC <sub>max</sub> )	7 $\pm$ 4	7 $\pm$ 4	18.6 (13.7 - 29.2)	0.89
<i>Slow 42°C</i>				
Absolute flux (PU)	25 $\pm$ 11	22 $\pm$ 10	21.1 (15.5 - 33.2)	0.30
Absolute CVC (PU/mmHg)	0.31 $\pm$ 0.14	0.28 $\pm$ 0.12	22.7 (16.7 - 35.7)	0.43
Maximal CVC (%CVC <sub>max</sub> )	10 $\pm$ 4	9 $\pm$ 5	24.7 (18.2 - 39.1)	0.73

**Table 2.** Local heating cutaneous blood flow results. Data is presented as mean  $\pm$  SD. Inter-day reproducibility is presented as CV ( $\pm$  95% CI): light grey shading indicates CV: >21%; mid grey shading indicates CV: 10-20%; dark grey shading indicates CV<10%.

	Trial 1	Trial 2	Between-day CV (%)	Paired t-test
<b>Plateau Phase (39°C /42°C)</b>				
<i>Rapid 39°C</i>				
Absolute flux (PU)	138 $\pm$ 56	150 $\pm$ 57	20.6 (15.1 - 32.3)	0.20
Absolute CVC (PU/mmHg)	1.76 $\pm$ 0.67	1.93 $\pm$ 0.76	21.2 (15.6 - 33.4)	0.22
Maximal CVC (%CVC <sub>max</sub> )	55 $\pm$ 16	57 $\pm$ 13	20.7 (15.2 - 32.6)	0.48
<i>Rapid 42°C</i>				
Absolute flux (PU)	233 $\pm$ 56	218 $\pm$ 45	13.1 (9.7 - 20.5)	0.15
Absolute CVC (PU/mmHg)	3.01 $\pm$ 0.73	2.80 $\pm$ 0.56	12.8 (9.5 - 20.0)	0.09
Maximal CVC (%CVC <sub>max</sub> )	87 $\pm$ 8	86 $\pm$ 7	6.8 (5.0 - 10.5)	0.56
<i>Gradual 42°C</i>				
Absolute flux (PU)	240 $\pm$ 35	242 $\pm$ 54	15.2 (11.2 - 23.7)	0.87
Absolute CVC (PU/mmHg)	3.00 $\pm$ 0.51	3.07 $\pm$ 0.69	15.9 (11.7 - 24.9)	0.69
Maximal CVC (%CVC <sub>max</sub> )	88 $\pm$ 4	90 $\pm$ 6	5.2 (3.9 - 8.1)	0.29
<i>Slow 42°C</i>				
Absolute flux (PU)	220 $\pm$ 47	231 $\pm$ 52	16.6 (12.3 - 26.0)	0.48
Absolute CVC (PU/mmHg)	2.61 $\pm$ 0.54	2.81 $\pm$ 0.63	18.2 (13.4 - 28.4)	0.26
Maximal CVC (%CVC <sub>max</sub> )	81 $\pm$ 11	87 $\pm$ 11	10.6 (7.8 - 16.5)	0.09
<b>Maximal Plateau (44°C)</b>				
<i>Rapid 39°C</i>				
Absolute flux (PU)	255 $\pm$ 49	261 $\pm$ 61	12.9 (9.5 - 20.0)	0.66
Absolute CVC (PU/mmHg)	3.22 $\pm$ 0.60	3.37 $\pm$ 0.82	13.2 (9.8 - 20.6)	0.38
<i>Rapid 42°C</i>				
Absolute flux (PU)	274 $\pm$ 62	254 $\pm$ 42	13.7 (10.1 - 21.3)	0.11
Absolute CVC (PU/mmHg)	3.46 $\pm$ 0.75	3.26 $\pm$ 0.55	12.3 (9.1 - 19.2)	0.14
<i>Gradual 42°C</i>				
Absolute flux (PU)	274 $\pm$ 42	275 $\pm$ 60	14.6 (10.8 - 22.8)	0.96
Absolute CVC (PU/mmHg)	3.39 $\pm$ 0.50	3.41 $\pm$ 0.74	14.5 (10.7 - 22.6)	0.93
<i>Slow 42°C</i>				
Absolute flux (PU)	274 $\pm$ 47	268 $\pm$ 54	13.2 (9.8 - 20.6)	0.71
Absolute CVC (PU/mmHg)	3.21 $\pm$ 0.58	3.23 $\pm$ 0.61	15.0 (11.1 - 23.5)	0.89

**Table 3.** Estimated sample sizes required for a repeated measures study design using the *Rapid 39°C*, *Rapid 42°C*, *Gradual 42°C* and *Slow 42°C* local heating protocols to detect significant changes in skin blood flow assessed as %CVC<sub>max</sub> at 39/42°C using the results from the reproducibility study.

Power, %	α-Error	Change in %CVC <sub>max</sub>	Sample size			
			<i>Rapid 39°C</i>	<i>Rapid 42°C</i>	<i>Gradual 42°C</i>	<i>Slow 42°C</i>
80	0.05	2.5	208	99	58	180
80	0.05	5.0	54	27	17	47
80	0.05	7.5	25	13	9	22
80	0.05	10.0	15	9	6	14
90	0.05	2.5	278	132	77	240
90	0.05	5.0	71	35	21	62
90	0.05	7.5	33	17	11	29
90	0.05	10.0	20	11	7	17

## FIGURE LEGENDS

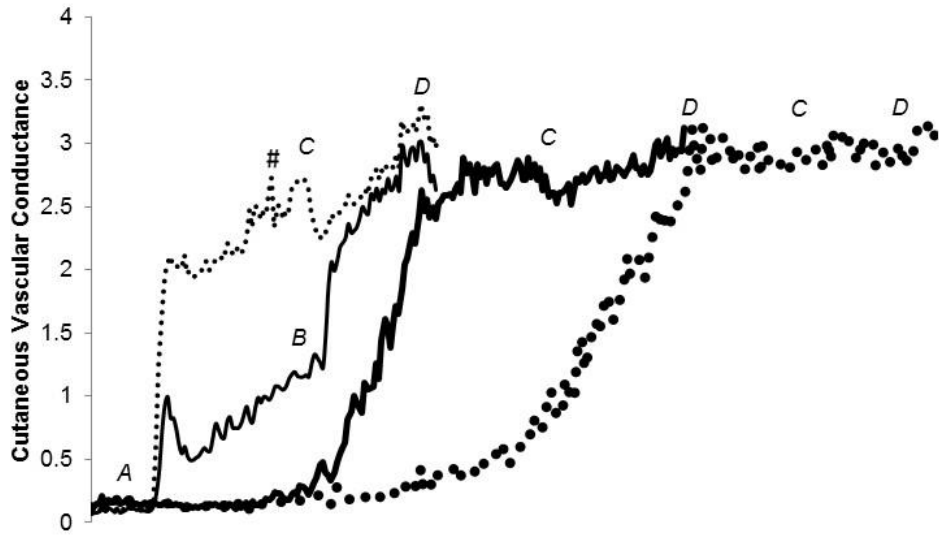
**Figure 1.A.** A representative forearm cutaneous arbitrary flux response for the four local heating protocols. Average flux was calculated over a stable 10-min period of baseline at 33°C (A) and the final 5-min of the plateau phases during heating at 39/42°C (B and C, respectively) and 44°C (D). The spike (#) indicates artefact resulting from slight movement of the arm which was removed prior to analysis. **B.** Step-wise temperature increments with corresponding times for all four local heating protocols.

**Figure 2.** Bland–Altman plots of the difference between days in %CVC<sub>max</sub> against the mean of the measurements at the 39/42°C plateau for **A.** *Rapid 39°C*, **B.** *Rapid 42°C*, **C.** *Gradual 42°C* and **D.** *Slow 42°C*. Middle horizontal line denotes mean value and upper and lower lines denote 95% limits of agreement. Linear regression demonstrated no evidence of proportional bias.

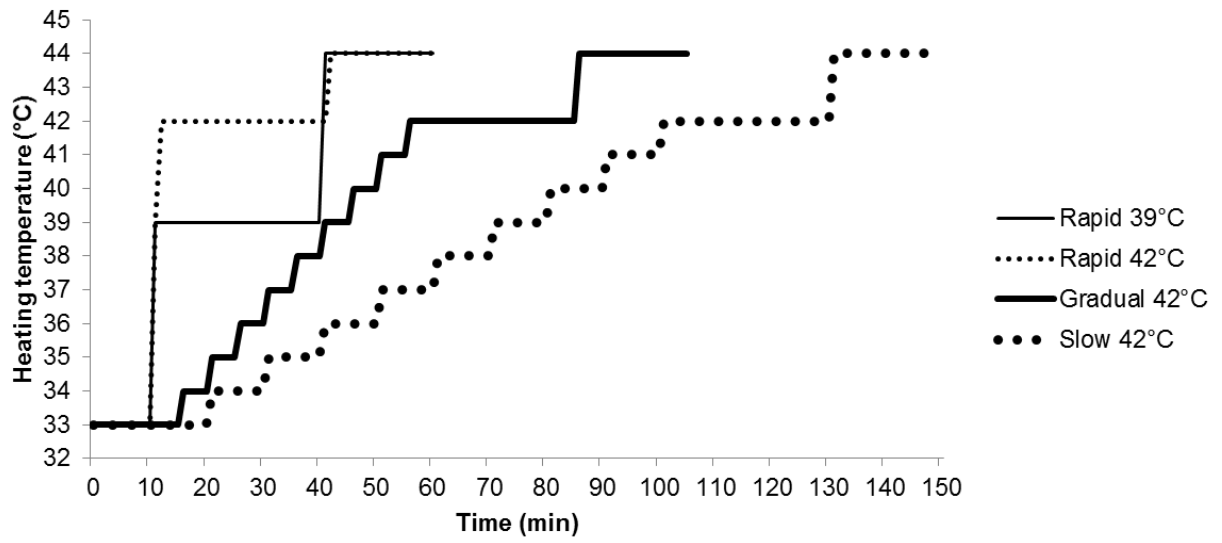
**Figure 3.** Bland–Altman plots of the difference between days in CVC against the mean of the measurements at 44°C for **A.** *Rapid 39°C*, **B.** *Rapid 42°C*, **C.** *Gradual 42°C* and **D.** *Slow 42°C*. Middle horizontal line denotes mean value and upper and lower lines denote 95% limits of agreement. Linear regression demonstrated no evidence of proportional bias.

Figure 1

A.

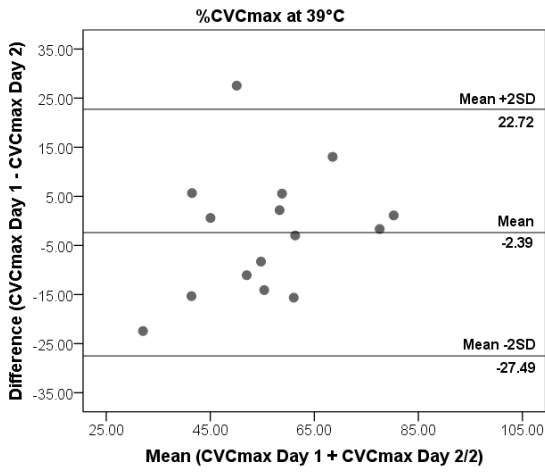


B.

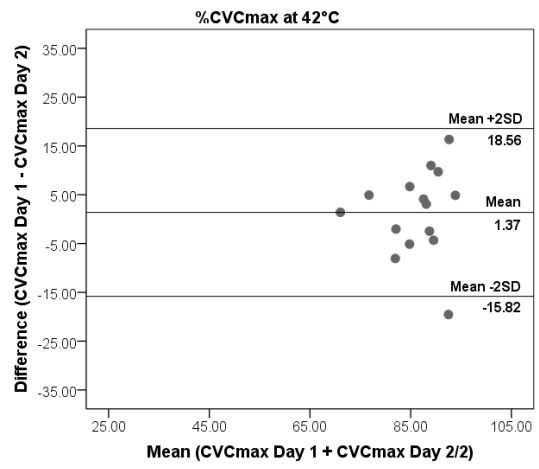




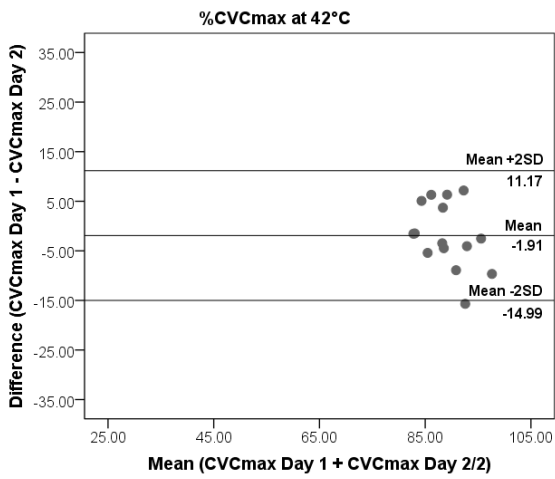
**Figure 2**



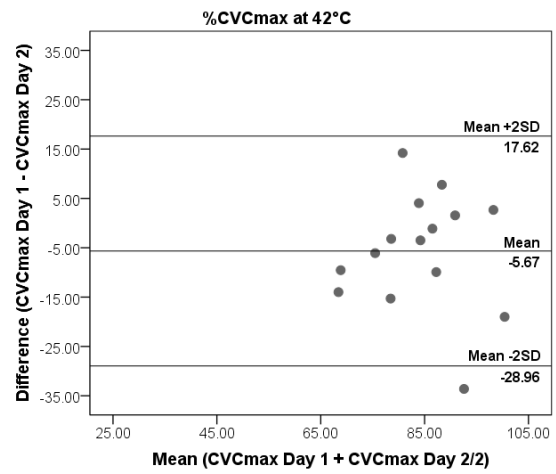
**A. Rapid 39°C**



**B. Rapid 42°C**

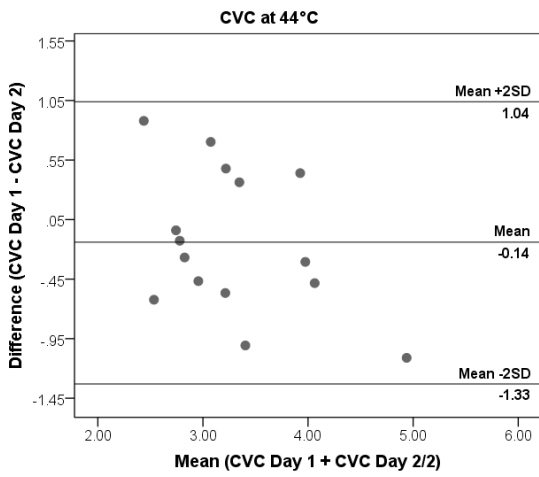


**C. Gradual 42°C**

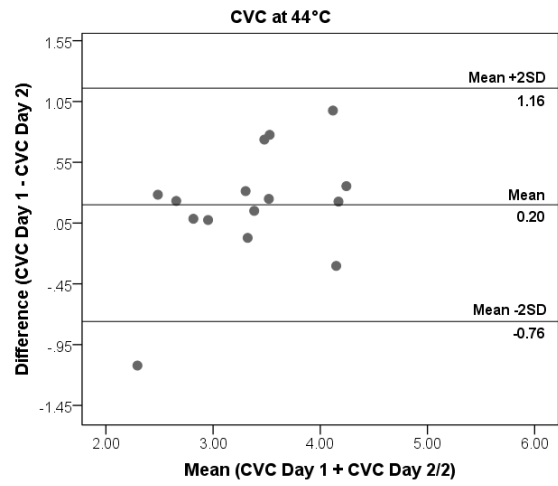


**D. Slow 42°C**

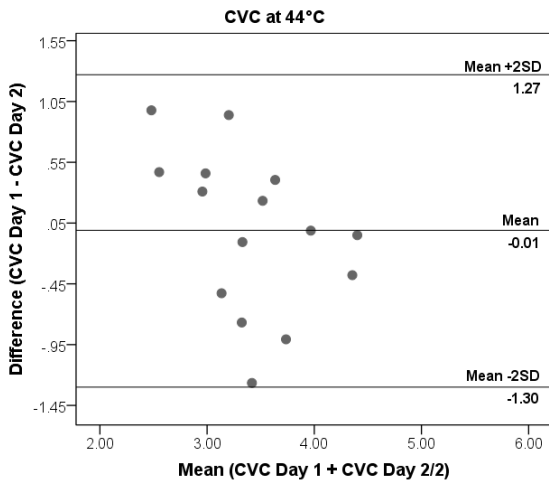
**Figure 3**



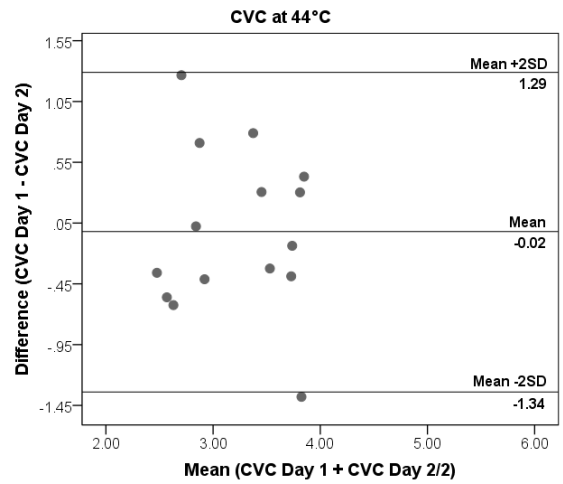
**A. Rapid 39°C**



**B. Rapid 42°C**



**C. Gradual 42°C**



**D. Slow 42°C**