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Tissue Viability Imaging: microvascular response to vasoactive drugs induced by iontophoresis

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Abstract

When one is studying the physiology of the cutaneous microcirculation there is a need for relevant non-invasive and versatile techniques. In this study we used a new optical device, the Tissue Viability Imager (TiVi), to map changes in cutaneous microvascular concentrations of red blood cells during iontophoresis of vasoactive substances (noradrenaline (NA) and phenylephrine (Phe) for vasoconstriction and acetylcholine (ACh) and sodium nitroprusside (SNP) for vasodilatation). We aimed to present data both individually and pooled, using a four-variable logistic dose response model that is commonly used in similar *in vitro* vascular studies. The accuracy of the TiVi was also investigated by calculating the coefficient of variation and comparing it with similar tests previously done using laser Doppler imaging. Tests were also performed using the TiVi and LDPI simultaneously to further compare the two methods.

Results showed that the TiVi is capable of quantifying vascular responses to iontophorised noradrenaline and phenylephrine without the need to increase background flow first.

Fitting the TiVi data to the dose response model resulted in ED_{50}-values with narrow confidence intervals and acceptable r^2 values. Mean ED_{50}-values for the TiVi did not differ significantly from similar values obtained using laser Doppler.

Results further seem to suggest that when the blood perfusion increases during vasodilatation in skin the initial phase relies mainly on an increase in red blood cell concentration whereas the further perfusion increase is due to an increase in red blood cell velocity.

Keywords: cutaneous microcirculation; iontophoresis; laser Doppler; tissue viability imager.

Introduction

In diseases such as diabetes and essential hypertension, reduction or loss of the regulatory function of the vascular system endothelium is thought to be an early key event (Adams, M.R. 2006). Efforts have therefore been made to develop improved *in vivo* methods that use the endothelial microvascular function as a biomarker in vascular disease. Microdoses of vasoactive substances can be given locally and non-invasively by iontophoresis into the cutaneous microcirculation with minimal systemic effects. This makes it a useful tool for assessment of vascular function, and several investigators have used the technique for this purpose (Morris, S.J. et al. 1995; Morris, S.J. and Shore, A.C. 1996; Noon, J.P. et al. 1998; Curdy, C. et al. 2001; Newton, D.J. et al. 2001; Henricson, J. et al. 2007). A number of substances can be given by iontophoresis but typically vasodilators, most commonly acetylcholine and sodium nitroprusside, have been used and the evoked vascular response has been measured by different laser Doppler techniques (Abularrage, C.J. et al. 2005). During iontophoresis, the electrical dose (defined as the product of duration and current strength), is thought to correlate with the amount of drug delivered through the skin (Phipps, J.B. et al. 1989; Droog, E.J. et al. 2004). If a sigmoidal curve, which is commonly used in pharmacological *in vitro* studies, is fitted to the variables of the vascular response such as ED_{50} and Hill slope, they can be quantified (Henricson, J. et al. 2007) which will facilitate the comparison of the effect of different drugs or the response of different groups of patients. Some limitations in the framework associated with assessment of vascular function by the laser Doppler technique are technically cumbersome for examining effects of vasoconstriction (Lipnicki, D.M. and Drummond, P.D. 2001).

We have used a Tissue Viability Imager (TiVi), a new tool for assessment of microvascular response to vasoactive stimuli. The TiVi uses linear polarisation light spectroscopy to gain

information about the red blood cell concentration (RBC_{conc}) in the cutaneous microvascular bed. The mean theoretical sampling depth in dermal tissue calculated by Monte Carlo simulations has been claimed to be 482 and 387 μm for the red and green wavelength regions, respectively (O'Doherty et al 2007). The actual depth of sampling is thought to be marginally larger - about 400-500 μm - while the epidermal layer is roughly 50-100 μm thick depending on its location in the body. This implies that the measurement volume (the volume from which the bulk of the signal is acquired) is located well into the reticular dermis in most skin sites.

Pilot studies have indicated that the TiVi-system can detect and quantify changes in tissue RBC_{conc} during both vasodilatation and vasoconstriction, and that the effect of vasoconstrictors can be quantified without the need of predilatation (O'Doherty, J. et al. 2007).

Our aim in the present study was therefore to investigate the ability of the TiVi-system to detect and quantify changes in the RBC_{conc} within the cutaneous microcirculatory network during iontophoresis of both vasoconstricting (noradrenaline (NA), phenylephrine (Phe)) and vasodilatating (ACh, SNP) substances. We hypothesised that ED_{50} values (the dose at which the RBC_{conc} is halfway between the initial and final value) could be calculated from the TiVi data using a four-variable logistic dose response model (Henricson, J. et al. 2007). A further aim was to compare data from vasodilatation studies generated by TiVi to data obtained from previous investigations using Laser Doppler perfusion imaging (LDPI) (Henricson, J. et al. 2007).

The methods were further compared by the simultaneous use of the TiVi and LDPI during iontophoresis of acetylcholine.

Methods

Subjects

Fourteen subjects (8 male) participated in the vasoconstriction study (mean age 33 years) and nine subjects (4 male) participated in the vasodilatation study (mean age 25 years). Eight subjects participated in the study using TiVi and LDPI simultaneously during iontophoresis of acetylcholine (all male, mean age 28 years). All studies were approved by the ethics committee at the Faculty of Health Science, Linköping University, Sweden. None of the subjects had any previous history of vascular disease and all gave informed consent to participate. All subjects were instructed not to drink tea or coffee or exercise 2 hours prior to the experiment and none had any ongoing medication (with the exception of oral contraceptives). All were non-smokers.

The subjects acclimatised for 20 minutes before of the experiments, resting on a bed with the left forearm supported on a pillow at heart level. The forearm skin was wiped gently with 70 % ethanol before the iontophoresis electrodes were attached. In all experiments the region of drug delivery on the forearm was chosen randomly. Areas with large underlying vessels, and signs of damaged skin, or both, were avoided. Ambient conditions (temperature about 21 degrees centigrade and room humidity about 30%) were kept as constant as possible throughout the experiment (Droog, E.J. et al. 2004; Henricson, J. et al. 2007; Tesselaar, E. 2007).

Demographic information and details of methods used in the vasodilatation study using ACh and SNP and LDPI are given in the paper by Henricson et al. (Henricson, J. et al. 2007).

Iontophoresis

A battery-powered iontophoresis controller (PeriIont 382, Perimed AB, Järfälla, Sweden) was used to deliver constant direct current pulses to the skin (Droog, E.J. et al. 2004).

A drug delivery silver, silver chloride electrode (Perimed AB, Järfälla, Sweden) with an internal diameter of 14 mm and a transparent plastic removable top was attached to the skin with adhesive tape. Another electrode that lacked the drug delivery capacity was also attached to the skin close to the wris to complete the circuit.

In the vasoconstriction tests the drug delivery electrode was filled with 430µl noradrenaline (NA) (1 mg/ml; Apoteket JJ Berzelius, Linköping, Vnr 33 82 36) or 430µl 1 mg/ml phenylephrine (Phe) (diluted with deionised water from 10 mg/ml; Apoteket JJ Berzelius, Linköping, Vnr 32 25 94). Both drugs were delivered by anodal iontophoresis.

In the vasodilatation studies, acetylcholine (ACh) (10 mg/ml; Miochol-E, Novartis Healthcare A/S, Copenhagen, Vnr 41 77 41) was given by anodal iontophoresis and sodium nitroprusside (SNP) (10 mg/ml; Nitropress, Hospira, Inc. Lake Forest, IL, USA, NDC 0409-3024-01) by cathodal iontophoresis.

In the tests using TiVi and LDPI simultaneously vascular responses were induced by anodal iontophoresis of acetylcholine (10 mg/ml).

All drugs were given using a constant current of 0.02 mA and a pulse duration of 10 minutes (charge density 7.8mC/cm^2, total electrical charge 12 mC) (Droog, E.J. et al. 2004). Before use the drugs were stored at room temperature and protected from light.

All drugs were delivered by the local hospital pharmacy (Apoteket J.J. Berzelius, Linköping University, 58185 Linköping, Sweden).

Tissue Viability Imager (TiVi)

Tissue RBC_{conc} was quantified by use of a commercial TiVi-system (TiVi600, WheelsBridge AB, Linköping, Sweden), which provides information about back-scattered light from the tissue and its microcirculation by using subsurface polarisation light spectroscopy. This system has recently been evaluated in separate studies with regard to performance (Nilsson, G.E. et al. 2009), ability to visualize skin erythema (Zhai, H. et al. 2009) and blanching (Zhai,

H. et al. 2009) as well as in the assessment of the skin barrier function (Wiren, K. et al. 2008). The TiVi-system consists of a digital camera (Digital Ixus 500, Canon, Japan) equipped with perpendicular polarisation filters (TechSpec Visible Linear Polarization Laminated Film, F45-668, Edmund Optics, UK) in front of the flash and lens. When the flash is activated, the broad spectrum white light emitted becomes linearly polarised after passage of the first polarisation filter. Reflected light from the skin consists of both polarised (directly reflected) and randomly polarised ("sub-surface") light. A second filter in front of the objective prevents any directly reflected light from reaching the photo-array in the camera. The RBCs in the microcirculation absorb light in the green wave-length region (about 500-600 nm) to a much higher extent than light in the red wave-length region (about 600-700 nm). The surrounding tissue components of the dermis, in comparison, absorb lesser light, and this absorption is not as wave-length 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 (red, green, and blue, see Figure 1.). A dedicated algorithm is applied that 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 wave-length region. The values of the resulting matrix, referred to as TiVi-values, scale linearly with the momentary RBC_{conc} in the actual tissue volume. Due to the selection of wavelength range the system is relatively insensitive to the oxygen state of the RBCs as confirmed by in-vitro experiments demonstrating a reduction in TiVi-values limited to about 10 % when saturation was reduced from 100 to 0 percent (O'Doherty, J. et al. 2007).

In contrast to techniques involving laser Doppler, the TiVi is insensitive to movement of the tissue and has the same degrees of freedom as standard photography. Further, it captures a full-format image instantaneously, thereby avoiding misinterpretation of temporal variability as spatial heterogeneity in the tissue RBC_{conc}. The maximum number of measurement points

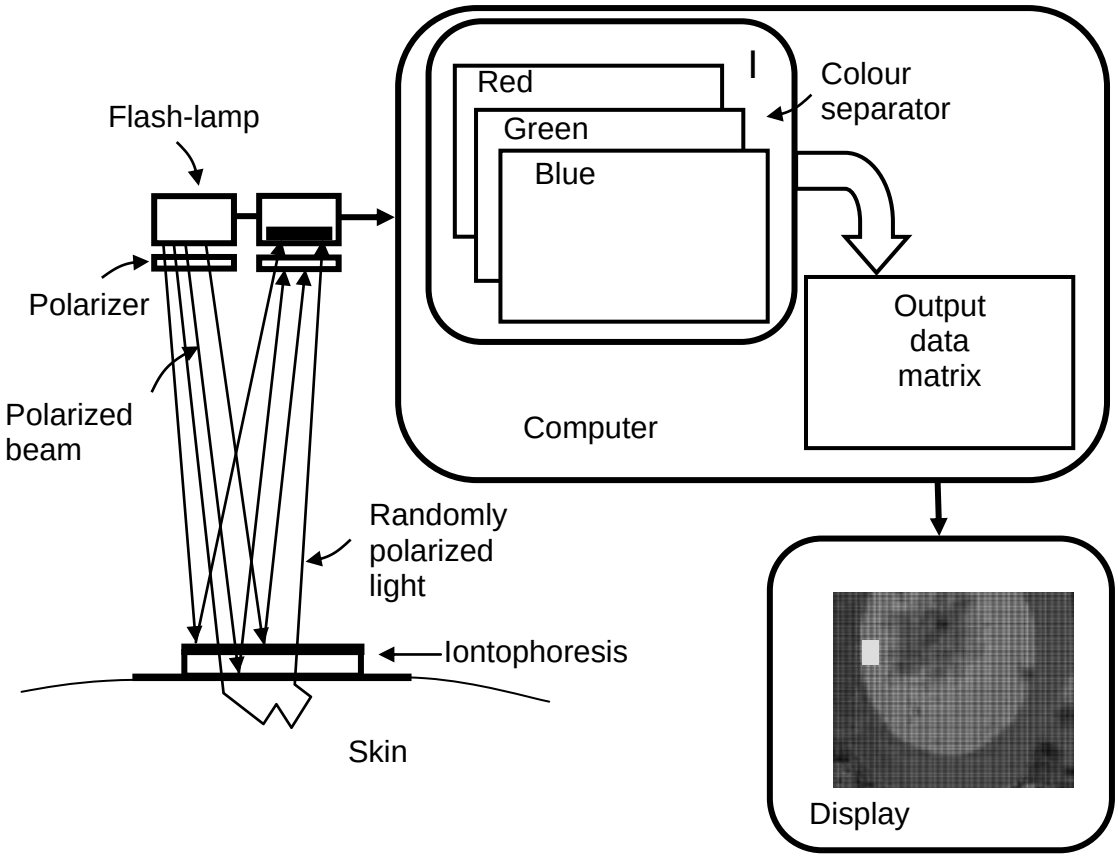

Figure 1: Schematic of the capturing and analysis of cutaneous microvascular data during iontophoresis of a vasoactive drug using the tissue viability imager (TiVi).

is limited only by the number of pixels recordable by the camera. Following occlusion of the blood flow to a limb, the TiVi-index is not approaching zero in the same way as the perfusion value recorded by laser Doppler techniques, because the TiVi system is sensitive only to the RBC_{conc} and not to the RBC velocity.

The distance between the camera and the iontophoresis electrode chamber area was about 7 cm during all vasoconstriction (NA and Phe) and vasodilatator (ACh and SNP) tests. The resolution was set to “small fine” (640 x 480 pixels) and the settings “flash on” and “macro” were used. Photographs were captured with 6 seconds intervals for 10 minutes and each test started with the recording of a baseline of 10 photographs. A total of 110 photographs were captured in each experiment.

Vascular responses to iontophorised acetylcholine and sodium nitroprusside recorded with LDPI and used for comparison with TiVi data in the present paper were recorded in a previous set of experiments (Henricson, J. et al. 2007).

Similar image size and camera settings as listed above were used also during the collection of images for the comparison study using the TiVi and LDPI simultaneously during iontophoresis of acetylcholine. Photographs were captured with a 10 second interval for 10 minutes. Each test started with the recording of a baseline of 10 photographs. A total of 70 photographs were captured in each experiment. The distance between the camera and test site was about 15 cm in all tests. During capture of a photograph the laser beam from the LDPI was cut off to avoid interference with the TiVi.

Image analysis was made using the analysis software (TiVi Version 2.1, WheelsBridge AB, Linköping, Sweden).

Data and statistical analysis

Generation of TiVi images

All TiVi-images were cropped and prepared for analysis in the same way. A region of interest (ROI) was selected corresponding to the area of the iontophoresis electrode chamber (1.5 cm^2) in every image. Images were aligned using a built- in feature of the TiVi-system software to ensure that all ROIs in a sequence of photographs covered exactly the same area. An average TiVi-value was calculated for each ROI, indicating the change in RBC_{conc} over the time course of the experiment.

For the experiments with ACh, SNP, NA and Phe every photographic sequence generated 110 mean RBC_{conc} values per subject and drug.

The obtained values were analysed in two ways: separately for each subject and each drug (**A**), and as pooled data of all values generated for each drug (**B**).

To these data we applied a four-variable logistic dose response model using a least squares curve fitting (Droog, E.J. et al. 2004; Henricson, J. et al. 2007).

(A) The individual curve fit data were normalised by using a mean of the baseline values and a mean of the last 10 TiVi values. Subjects that did not respond to the treatment with a significant decrease compared with baseline (Student's *t* test) were considered "non-responders" and their data excluded. Curves that lacked stable plateaus, and consequently resulted in an inaccurate curve fit, were excluded from further individual calculations.

(B) Values from all subjects, except non-responders, were included in the calculation and analysis of the pooled graph, even if no plateau was reached for the last images in a sequence. These data were normalised in a similar way as described for the individual curve fits.

For the comparison study using the TiVi and LDPI simultaneously every photographic sequence generated 70 images that were cropped and prepared for analysis as described above. Mean TiVi-values were plotted as a function of LDPI-values. Significant response to the treatment was tested for in a similar way as previously described. Individual Pearson product-moment correlation coefficients (PMCC) were calculated for each subject as well as the PMCC for the pooled data.

Generation of laser Doppler images

Procedures for the calculations and data analysis for the vascular response to ACh and SNP obtained by LDPI used for comparison of ED_{50} values and calculations of CV with similar data collected by the TiVi have previously been published by Henricson et al. (Henricson, J. et al. 2007).

Images for the comparison study using the TiVi and LDPI simultaneously were collected by using a laser Doppler perfusion imager (Perimed PIM 2.0, Järfälla, Sweden) with the following settings; medium resolution, medium scan speed, threshold value 6.2 V, and 10 x 10 pixels image size. A full scan was completed every 10^{th} second and was performed in between capture of TiVi images. All lights in the room were turned off during measurements.

Student's *t* test was used for comparison between groups, and p-values less than 0.05 were accepted as statistically significant in all calculations. Data points in graphs are presented as mean values and error bars as standard error of the mean (SEM).

The following software were used for calculations and curve fits: Microsoft Office Excel (2003 SP2), (Microsoft Corporation) and GraphPad Prism (Prism 4 for Windows, Version 4.00 April, 2003).

Results

Vasoconstriction (TiVi)

Noradrenaline

Individual curve fit. The results from the individual curve fits (n=10) are shown in Table 1a. The mean ED_{50} was 4.4 mC with a 95% confidence interval of 3.2 to 5.7 mC and a mean r^2 of

Table 1: Results from the individual curve fit of the mean responses to noradrenaline 1 mg/ml (Table 1a) and phenylephrine (Table 1b) (n=10 respectively). Vascular response was measured using the tissue viability imager (TiVi).

Table 1a. (TiVi)	**Noradrenaline (1 mg/ml)**		
Subject (n=10)	**ED_{50} (mC)**	**95 % CI of ED_{50}**	**r^2**
1	4.3	3.7 to 5.1	0.85
2	3.0	2.1 to 4.3	0.50
3	2.8	2.3 to 3.4	0.58
5	4.5	4.0 to 5.0	0.96
8	4.2	3.3 to 5.3	0.71
9	5.6	4.6 to 6.8	0.76
10	1.6	1.0 to 2.7	0.38
11	7.6	5.9 to 9.8	0.63
12	4.8	4.2 to 5.5	0.86
13	6.0	5.2 to 7.0	0.98
Mean	**4.4**	**3.2 to 5.7**	**0.72**

Table 1b. (TiVi)	**Phenylephrine (1 mg/ml)**		
Subject (n=10)	**ED_{50} (mC)**	**95 % CI of ED_{50}**	**r^2**
1	1.5	1.3 to 1.7	0.87
2	6.8	5.6 to 8.3	0.77
4	7.8	5.5 to 11	0.91
5	4.4	3.5 to 5.5	0.92
7	1.5	1.1 to 2.0	0.81
9	5.6	4.8 to 6.5	0.70
10	6.6	5.3 to 8.2	0.82
12	3.7	3.4 to 4.0	0.81
13	5.5	5.4 to 5.7	0.99
14	5.1	4.6 to 5.7	0.91
Mean	**4.9**	**3.3 to 6.4**	**0.90**

0.72. Two subjects did not reach stable response plateaus and data could therefore not be fitted to the sigmoidal analysis model. Two subjects did not respond with a significant decrease in RBC_{conc} and the data were therefore excluded from further calculations.

Curve fit of pooled graphs. The pooled vascular RBC_{conc} response to NA (n=12) is shown in Figure 2a. The ED_{50} value for the pooled data was 5.0 mC with a 95 % CI of 4.6 to 5.4 mC and an r^2 value of 0.98.

Phenylephrine

Individual Curve fit. The results of the individual curve fits (n=10) for the RBC_{conc} data in response to Phe are shown in Table 1b. The mean ED_{50} was 4.9 mC with a 95% confidence interval of 3.3 to 6.4 mC and a mean r^2 of 0.90. Three subjects were non-responders and 1 did not reach a stable response plateau, data from these subjects were therefore excluded from further individual curve fitting.

Curve fit on pooled data. The pooled vascular RBC_{conc} response to Phe (n=13) is shown in Figure 2b. The ED_{50} value for the pooled data was 5.0 mC with a 95 % CI of 4.7 to 5.4 mC and an r^2 value of 0.99.

Comparison between noradrenaline and phenylephrine

Individual Curve fit. ED_{50} values obtained from the individually fitted curves for NA and Phe using the TiVi showed no significant difference (p=0.64).

Coefficient of variation. The coefficient of variance for the mean ED_{50} value generated by individual curve fits is shown in Table 2a.

Curve fit on pooled data. No significant difference between the two drugs could be found for the ED_{50} values gained from the pooled data (p =0.88).

Coefficient of variation. The CV for the ED_{50} value from the pooled vasoconstriction data is shown in Table 2b.

Figure 2a.
Noradrenaline [1 mg/ml]

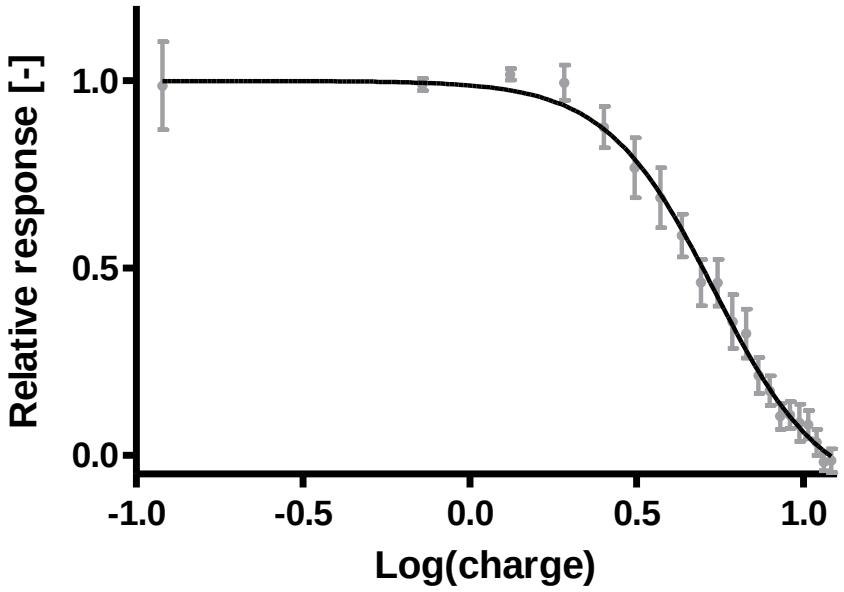

Figure 2b.
Phenylephrine [1 mg/ml]

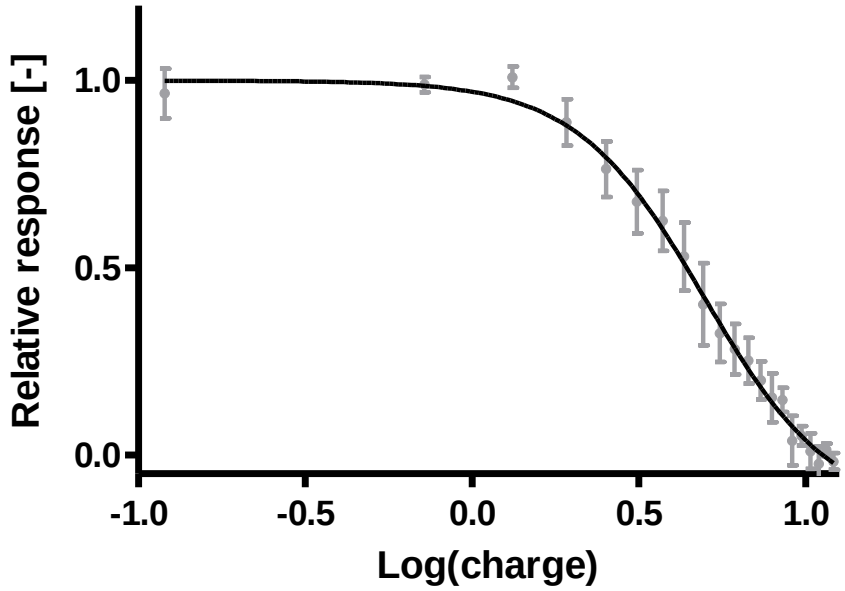

Figure 2: Graph of the pooled mean RBC_{conc} response to iontophoretically given noradrenaline 1 mg/ml (1a) and phenylephrine (1b) given iontophoretically in 12 and 11 subjects respectively, measured by the tissue viability imager (TiVi). Error bars are shown as SEM.

Table 2: Table 2a shows the coefficient of variation for the ED_{50} values obtained after individual curve fitting using the tissue viability imager (TiVi). Table 2b shows the corresponding calculation for the pooled mean ED_{50} value.

Table 2a. **TiVi**

ED_{50} (mC)	**Acetylcholine (n=7)**	**Sodium nitroprusside (n=6)**	**Noradrenaline (n=10)**	**Phenylephrine (n=10)**
Mean	2.8	4.9	4.4	4.9
SD	1.1	2.0	1.7	2.1
SEM	0.4	0.8	0.5	0.7
Range	1.8 to 5.0	2.1 to 7.4	1.6 to 7.6	1.5 to 7.8
95 % CI	1.9 to 3.8	2.7 to 6.8	3.2 to 5.7	3.3 to 6.4
CV (%)	37.3	41.4	39.0	44.0

Table 2b. **TiVi**

ED_{50} (mC)	**Acetylcholine (n=7)**	**Sodium nitroprusside (n=7)**	**Noradrenaline (n=12)**	**Phenylephrine (n=11)**
Mean	2.8	4.4	4.4	4.6
SD	1.1	2.0	1.7	2.2
SEM	0.4	0.76	0.5	0.67
Range	1.8 to 5.0	2.1 to 7.4	1.6 to 7.6	1.5 to 7.8
95 % CI	1.9 to 3.8	2.6 to 6.3	3.2 to 5.7	3.1 to 6.1
CV (%)	37.3	45.4	39.1	48.8

Vasodilatation (TiVi and LDPI)

Acetylcholine (TiVi)

Individual curve fits. The results from the curve fits of the RBC_{conc} data in response to ACh are shown in Table 3a. The mean ED_{50} was 2.8 mC with a 95% confidence interval of 1.9 to 3.8 mC and a mean r^2 of 0.75. One subject was classified as non-responder and data were excluded from further individual curve fitting.

Table 3: Results from the individual curve fit and mean responses to acetylcholine 10 mg/ml (Table 3a) and sodium nitroprusside (Table 3b) (n=7, n=6 respectively). Vascular response was measured using the tissue viability imager (TiVi).

Table 3a. (TiVi)	**Acetylcholine (10 mg/ml)**		
Subject (n=7)	**ED_{50} (mC)**	**95 % CI of ED_{50}**	**r^2**
2	2.7	2.4 to 2.9	0.82
3	2.1	1.9 to 2.2	0.85
4	1.8	1.6 to 2.0	0.47
5	2.8	2.5 to 3.3	0.64
6	5.0	4.7 to 5.3	0.88
7	2.3	2.1 to 2. 5	0.69
8	3.3	3.1 to 3.5	0.92
Mean	**2.8**	**1.9 to 3.8**	**0.75**

Table 3b. (TiVi)	**Sodium nitroprusside (10 mg/ml)**		
Subject (n=6)	**ED_{50} (mC)**	**95 % CI of ED_{50}**	**r^2**
1	2.1	1.9 to 2.4	0.63
2	5.6	5.2 to 6.1	0.80
4	3.0	2.9 to 3.2	0.93
5	7.4	7.1 to 7.7	0.89
7	4.5	3.9 to 5.2	0.52
8	6.0	5.5 to 6.6	0.67
Mean	**4.9**	**2.7 to 6.8**	**0.74**

Curve fit on pooled data. The graph for the pooled RBC_{conc} data in response to ACh is shown in Figure 3a. The ED_{50} value for the pooled RBC_{conc} data was 2.8 mC with a 95 % CI of 2.6 to 3.1 mC and an r^2 value of 0.97.

Sodium nitroprusside (TiVi)

Individual curve fits. The detailed individual data on the SNP responses are presented in Table 3b. The mean ED_{50} was 4.9 mC with a 95% confidence interval of 2.7 to 6.8 mC and a

Figure 3a.
Acetylcholine [10 mg/ml]

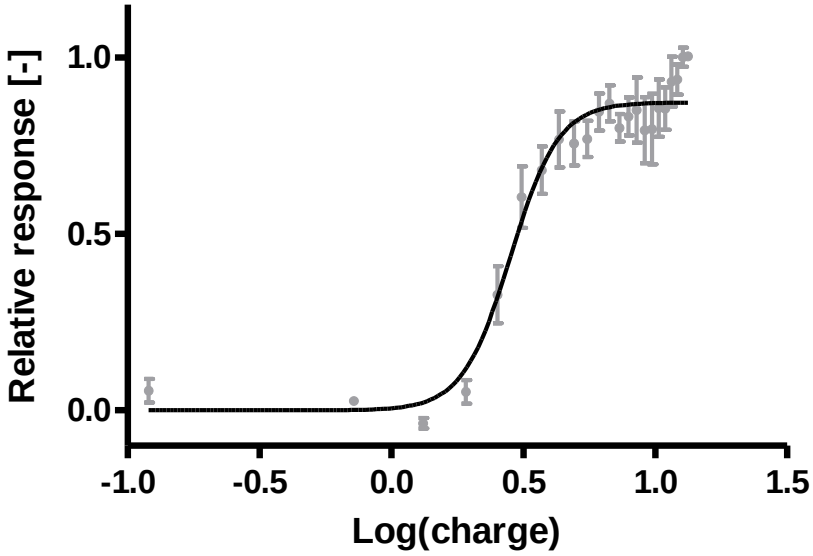

Figure 3b.
Sodium nitroprusside [10 mg/ml]

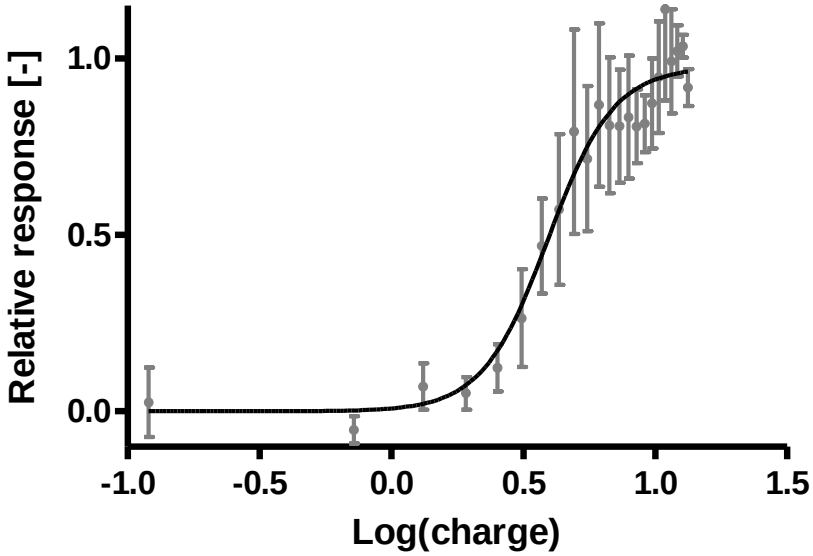

Figure 3: Graph of the pooled mean RBC_{conc} response to iontophoretically given acetylcholine (3a) 10 mg/ml and sodium nitroprusside (3b) in 7 subjects respectively, measured by the tissue viability imager (TiVi). Error bars are shown as SEM.

mean r^2 of 0.74. Data from two subjects were excluded due to lack of response and lack of a stable maximum perfusion plateau, respectively.

Curve fit on pooled data. The ED_{50} value for the pooled RBC_{conc} data from the SNP tests was 3.9 mC with a 95 % CI of 3.5 to 4.4 mC and a mean r^2 of 0.96. Results are shown in Figure 3b.

Comparison between acetylcholine and sodium nitroprusside

Individual Curve fits. Mean ED_{50} value for ACh and SNP using the TiVi and individual curve fits differed significantly (p=0.048).

Coefficient of variation. The CV of the mean ED_{50} value from the vasodilatation tests using the individual curve fit is shown in Table 3a.

Pooled Curve fits. The ED_{50} values for the pooled ACh and SNP data using the TiVi differed significantly (p=0.023).

Coefficient of variation. The CV for the ED_{50} value from the pooled vasodilatation data is shown in Table 3b.

Comparison of methods (TiVi and LDPI)

Acetylcholine

Individual Curve fits. Mean ED_{50} values for TiVi and LDPI measurements using ACh and individual curve fitting showed no significant difference (p value = 0.16).

Coefficient of variation. The coefficient of variation for the mean ED_{50} value from the individual curve fitting using the TiVi was 39 % and the corresponding value using LDPI was 38 %.

Pooled Curve fits. The ED_{50} values for the pooled ACh data using TiVi and LDPI showed no significant difference (p= 0.12).

Coefficient of variation. The coefficient of variation for the ED_{50} value for the pooled data of the ACh group measured by TiVi was 39 % and the corresponding value for the experiments using LDPI was 38 %.

Sodium nitroprusside

Individual Curve fits. Mean ED_{50} values for TiVi and LDPI measurements using SNP and individual curve fitting showed no significant difference ($p = 0.45$).

Coefficient of variation. The coefficient of variation for the mean ED_{50} value from the individual curve fitting using the TiVi was 44 % and the corresponding value using LDPI was 28 %.

Pooled Curve fits. The ED_{50} values for the pooled SNP data using TiVi and LDPI showed no significant difference ($p = 0.45$).

Coefficient of variation. The coefficient of variation for the ED_{50} value for the pooled data of the SNP group measured by TiVi was 49 % and the corresponding value for the experiments using LDPI was 28 %.

Simultaneous measurement using TiVi and LDPI

Figure 4 shows the average TiVi-values plotted as a function of average LDPI-values recorded during iontophoresis of 10 mg/ml acetylcholine. The correlation coefficient was calculated to 0.85.

The individual correlation coefficients for the participating subjects (n=8) are presented in table 4.

Figure 4.
Acetylcholine [1mg/ml]

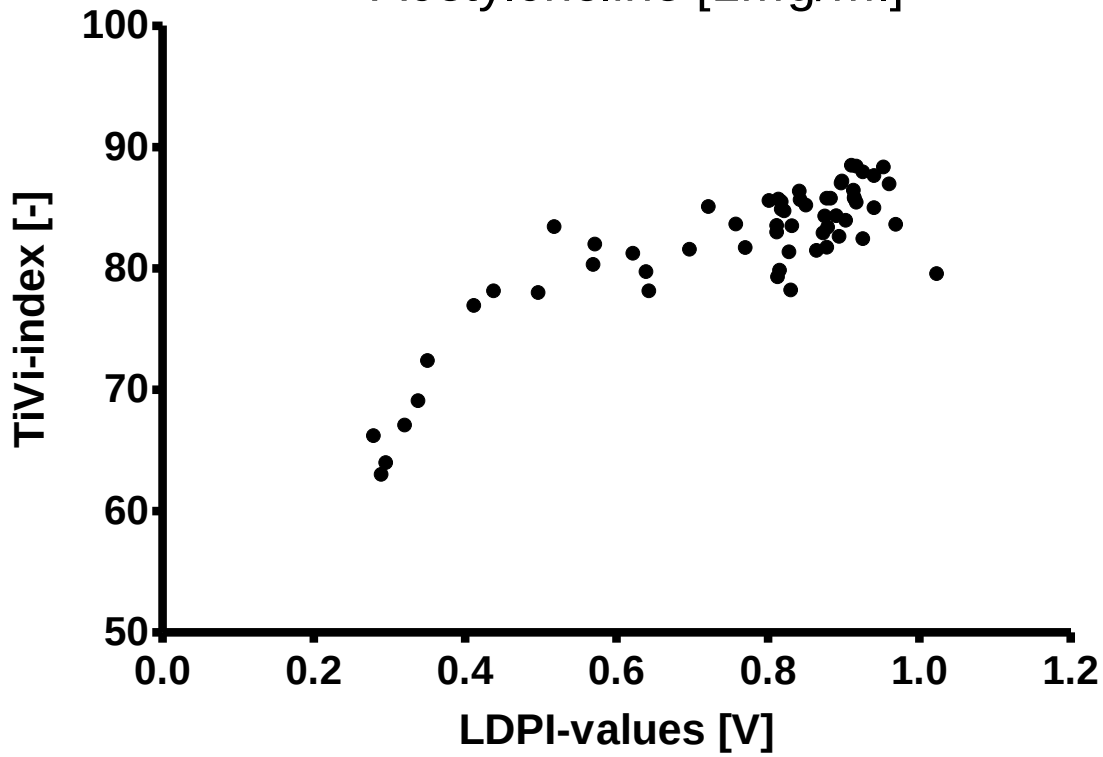

Figure 4: The simultaneously recorded average TiVi-values plotted as a function of the average LDPI-values (n=8). The correlation coefficient was calculated to 0.85.

Table 4: The individual correlation coefficients for the TiVi-values plotted as a function of LDPI-values.

Acetylcholine [10 mg/ml]	
Subject	**Pearson r**
1	0.68
2	0.32
3	0.43
4	0.70
5	0.75
6	0.83
7	0.31
8	0.49

Discussion

We measured cutaneous vascular responses elicited by iontophoresis of vasoconstricting agents (noradrenaline and phenylephrine) and vasodilatating agents (acetylcholine and sodium nitroprusside) using a new optical measurement technique - the Tissue Viability Imager. Data were analysed using a four-variable-logistic-dose-response model in accordance to a previously developed protocol using laser Doppler (Droog, E.J. et al. 2004; Henricson, J. et al. 2007). The results show that the TiVi generates reproducible data that can be fitted into the proposed dose-response analysis model with ED_{50}-values that do not differ significantly from corresponding values obtained using laser Doppler. We also conclude that the TiVi is able to quantify vascular responses to iontophoresis of noradrenaline and phenylephrine without the need of increasing background skin blood flow first. The results further seem to suggest that when the blood perfusion increases in skin the initial phase is dependent mainly on the change in RBC concentration, which is followed by an increase in velocity of the RBCs as perfusion further increases.

Microvascular beds with few and slow-moving RBCs, such as the upper dermis of the forearms, oppose a challenge to laser Doppler techniques as the perfusion values (defined as the product of the mean RBC velocity and the number of RBCs) are typically low or close to the biological zero. A further reduction in blood flow induced for example by a vasoconstrictor is therefore difficult to detect. To circumvent this problem, background flow is often increased to enhance contrast, most commonly by local warming (Lipnicki, D.M. and Drummond, P.D. 2001; Drummond, P.D. 2002). However, findings of Wilson et. al. (Wilson, T.E. et al. 2002) suggest that local heating reduces vasoconstrictor responses to exogenous noradrenaline, specifically in non-glabrous skin. A dose of noradrenaline, which under normal

conditions of temperature will induce a clear vasoconstrictor response, was insufficient to overcome the influence of cutaneous vasodilatation completely by local warming

The TiVi measures the RBC_{conc} in tissue, and is therefore insensitive to the velocity of the RBCs. As the RBC_{conc} is primarily sensitive to the calibre and haematocrit of the vessels under investigation (Silverman, D.G. et al. 1994) assessment of vascular function with the TiVi resembles in vitro vessels studies in organ baths. This feature of the TiVi also allows for vasoconstriction studies on vessels in their normal unprovoked state without the need of predilatation (O'Doherty, J. et al. 2007), which reduces the number of confounding factors. This ability of the TiVi system was tested by iontophoresis of noradrenaline (α_1- and α_2-agonist) and phenylephrine (α_1-agonist) on the forearms of 14 healthy volunteers.

In areas dominated by nutritive flow, such as the forearm skin, baseline flow-flux is relatively low and and therefore unlikely to be dramatically affected by application of a vasoconstricting stimulus (Gardner-Medwin, J.M. et al. 1997; Brown, H. et al. 2003). Despite this a decrease in RBC_{conc} could still be seen in all subjects following iontophoresis of the vasoconstrictors. However, five subjects (2 for NA and 3 for Phe) never reached stable response plateaus or responses that were significantly different from baseline values. Although only one subject (subject 6) failed to reach a significant response during both measurements for noradrenaline and phenylephrine, subjects with a non-response or lack of plateau in response to one of the drugs showed lower values also in the response to the other drug.

A similar relationship between baseline values and TiVi-index at the end of the treatment could be seen in the measurements using the TiVi during iontophoresis of acetylcholine and sodium nitroprusside. The single non-responder during the measurement using acetylcholine had the highest mean TiVi-index (239, arbitrary units) for the baseline values of the entire group of 8 subjects (second highest mean TiVi-index; 153). The subjects that were excluded from the SNP measurements showed the highest and third highest mean TiVi-index for the

baseline, 242 and 186 respectively. The subject in between these non-responders showed a significant change in RBC_{conc} between baseline and end values, however, compared to the responses of the rest of the group the change was less pronounced.

These results seem to reflect the effect of initial tonus of the vessel bed on the responsiveness to iontophoretically administrated ACh, SNP, NA and Phe. The activity of the cutaneous microvessels is known to vary considerably between individuals and tonus is influenced by variations in, for example, vasomotor activity, mental state and hormonal levels – factors that are difficult to fully control for.

It is possible that the number of responders could be improved by the use of provocations prior the experiments inducing vasodilatation (e.g. local warming) and constriction (e.g. local cooling) to enhance the contrast. Pre-contraction or pre-relaxation of vessels is common procedure during *in vitro* strain gauge studies of vessels. In the present study the advantages of such a procedure have to be weighed against the possible disadvantages of introducing yet another step into an already highly complex *in vivo* model and further complicate the interpretation of data.

A weak response could further be the result of insufficient concentration of drug within the test site. As Tesselaar (Tesselaar, E. 2007) described, the response to an iontophoretically applied drug is affected by clearance of drug ions from the skin by passive diffusion and washout by blood flow. Competition between drug ions and other charged substances present either in the solution in the electrode chamber or in the body, reduces the amount of active substance that reaches the target receptors. The amount of drug delivered during iontophoresis is also affected by physiological factors with high interindividual variations such as skin thickness and skin pH. Increasing the availability of the drug by using a higher total electrical charge could lead to a greater number of accurate curve fits. However, the total electrical charge is limited because of the risk of non-specific vasodilatation.

A comparison of results obtained by the TiVi and Laser Doppler Imaging data seems to suggest that there is a curvilinear relationship between the two parameters with a break point at LDPI values around 0.5 (TiVi-values around 80) approximately at the normal skin balance point. The curvilinear relationship between LDPI and the TiVi values further suggests that when the blood perfusion increases, the initial phase is dependent on RBC_{conc} change rather than the velocity component. After this phase, further increases in perfusion seem to be caused by increases in RBC velocity.

It may be debated what technique to use to analyse the data; either as pooled data of all values generated from several subjects or as separate curve fitting in each subject. In this paper we present both options. Interestingly, there seems to be a difference – albeit minor - between these two techniques. If the number of negative influences on the curve fitting technique could be reduced the individual curve fitting procedure may be claimed to be more accurate, and it is also similar to the technique commonly used for "in vitro" experiments of vascular reactivity.

The present experimental model (iontophoresis and logistic dose response model fitting) looks promising for future clinical applications where for example the effect on the endothelium by different diseases is to be investigated by use of, for example, acetylcholine-/sodium nitroprusside-induced vasodilatation or the sensitivity to vasoconstrictive α-agonists. The rather restricted confidence intervals (ED_{50}) generated both for vasoconstriction and vasodilatation and their reproducibility between different experimental groups (in the present investigation healthy volunteers) supports the use of this technique in experimental medicine.

References:

Abularrage, C.J., Sidawy, A.N., Aidinian, G., Singh, N., Weiswasser, J.M. and Arora, S. 2005. Evaluation of the microcirculation in vascular disease. J Vasc Surg 42(3). 574-81.

Adams, M.R. 2006. Clinical assessment of endothelial function. Endothelium 13(6). 367-74.

Brown, H., Moppett, I.K. and Mahajan, R.P. 2003. Transient hyperaemic response to assess vascular reactivity of skin: effect of locally iontophoresed acetylcholine, bradykinin, epinephrine and phenylephrine. Br J Anaesth 90(4). 446-51.

Curdy, C., Kalia, Y.N. and Guy, R.H. 2001. Non-invasive assessment of the effects of iontophoresis on human skin in-vivo. J Pharm Pharmacol 53(6). 769-77.

Droog, E.J., Henricson, J., Nilsson, G.E. and Sjoberg, F. 2004. A protocol for iontophoresis of acetylcholine and sodium nitroprusside that minimises nonspecific vasodilatory effects. Microvasc Res 67(2). 197-202.

Drummond, P.D. 2002. Prior iontophoresis of saline enhances vasoconstriction to phenylephrine and clonidine in the skin of the human forearm. Br J Clin Pharmacol 54(1). 45-50.

Gardner-Medwin, J.M., Taylor, J.Y., Macdonald, I.A. and Powell, R.J. 1997. An investigation into variability in microvascular skin blood flow and the responses to transdermal delivery of acetylcholine at different sites in the forearm and hand. Br J Clin Pharmacol 43(4). 391-7.

Henricson, J., Tesselaar, E., Persson, K., Nilsson, G. and Sjoberg, F. 2007. Assessment of microvascular function by study of the dose-response effects of iontophoretically applied drugs (acetylcholine and sodium nitroprusside)--methods and comparison with in vitro studies. Microvasc Res 73(2). 143-9.

Henricson, J., Tesselaar, E., Persson, K., Nilsson, G. and Sjoberg, F. 2007. Assessment of microvascular function by study of the dose-response effects of iontophoretically applied drugs (acetylcholine and sodium nitroprusside)-Methods and comparison with in vitro studies. Microvasc Res 73(2). 143-9.

Lipnicki, D.M. and Drummond, P.D. 2001. Facilitating laser Doppler measurements of cutaneous adrenergic vasoconstriction: a comparison of methods. Clin Auton Res 11(2). 93-8.

Morris, S.J. and Shore, A.C. 1996. Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: possible mechanisms. J Physiol 496 (Pt 2). 531-42.

Morris, S.J., Shore, A.C. and Tooke, J.E. 1995. Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. Diabetologia 38(11). 1337-44.

Newton, D.J., Khan, F. and Belch, J.J. 2001. Assessment of microvascular endothelial function in human skin. Clin Sci (Lond) 101(6). 567-72.

Nilsson, G.E., Zhai, H., Chan, H.P., Farahmand, S. and Maibach, H.I. 2009. Cutaneous bioengineering instrumentation standardization: the Tissue Viability Imager. Skin Res Technol 15(1). 6-13.

Noon, J.P., Walker, B.R., Hand, M.F. and Webb, D.J. 1998. Studies with iontophoretic administration of drugs to human dermal vessels in vivo: cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. Br J Clin Pharmacol 45(6). 545-50.

O'Doherty, J., Henricson, J., Anderson, C., Leahy, M.J., Nilsson, G.E. and Sjoberg, F. 2007. Sub-epidermal imaging using polarized light spectroscopy for assessment of skin microcirculation. Skin Res Technol 13(4). 472-84.

Phipps, J.B., Padmanabhan, R.V. and Lattin, G.A. 1989. Iontophoretic delivery of model inorganic and drug ions. J Pharm Sci 78(5). 365-9.

Silverman, D.G., Jotkowitz, A.B., Freemer, M., Gutter, V., O'Connor, T.Z. and Braverman, I.M. 1994. Peripheral assessment of phenylephrine-induced vasoconstriction by laser Doppler flowmetry and its potential relevance to homeostatic mechanisms. Circulation 90(1). 23-6.

Tesselaar, E. (2007). Assessment of microvascular function by use of transdermal iontophoresis: methodological aspects. Linköping University, Department of Medicine and Care.

Tesselaar, E. (2007). Assessment of microvascular function by use of transdermal iontophoresis; methodological aspects. Department of Medicine and Care and Department of Biomedicine and Surgery, Faculty of Health Sciences. Linköping, Linköpings Universitet**:** No. 1002;64.

Wilson, T.E., Cui, J. and Crandall, C.G. 2002. Effect of whole-body and local heating on cutaneous vasoconstrictor responses in humans. Auton Neurosci 97(2). 122-8.

Wiren, K., Frithiof, H., Sjoqvist, C. and Loden, M. 2008. Enhancement of bioavailability by lowering of fat content in topical formulations. Br J Dermatol.

Zhai, H., Chan, H.P., Farahmand, S., Nilsson, G.E. and Maibach, H.I. 2009. Comparison of tissue viability imaging and colorimetry: skin blanching. Skin Res Technol 15(1). 20-3.

Zhai, H., Chan, H.P., Farahmand, S., Nilsson, G.E. and Maibach, H.I. 2009. Tissue viability imaging: mapping skin erythema. Skin Res Technol 15(1). 14-9.