METHOD OPTIMISATION AND VALIDATION STUDY ON USE OF LASER DOPPLER FLOWMETRY TO ASSESS MICROVASCULAR ENDOTHELIAL FUNCTION

LAPORAN AKHIR GERAN JANGKA PENDEK

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MARCH 2004

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A. MAKLUMAT AM

Tajuk Projek:
Method Optimisation and Validation Study on use of Laser Doppler Flowmetery to Assess Microvascular Endothelial Function

Tajuk Program:

Tarikh Mula: March 2003

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☐ **Rekaan atau perkembangan produk baru,**
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(1)

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(1) Projek ini bertujuan mengoptimasikan metodologi/kaedah untuk mengukur kesihatan salurdarah kecil (microvascular) dalam badan manusia. Kaedah yang menggunakan alat yang dipanggil laser doppler flowmetry dan proses hyperemia

reaktif telah berjaya dioptimasikan. Di samping itu, validasi kaedah dari segi 'intraday dan interday reproducibility' telah menunjukkan reproducibility yang baik bagi kaedah ini. Peningkatan pengetahuan dan penambahbaikan kaedah mengukur kesihatan salurdarah mikro dalam manusia telah berjaya dicapai melalui projek ini. Kaedah tidak invasif ini adalah suatu kaedah yang berpotensi untuk mengenalpasti petanda awal penyakit kardiovaskular manusia.

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(1) _____

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G. PENERBITAN HASIL DARIPADA PROJEK

(i) LAPORAN/KERTAS PERSIDANGAN ATAU SEMINAR

(1) Dua [2] kertas kerja hasil projek dibentangkan pada persidangan Antarabangsa: ' 20th Scientific Meeting of the International Society of Hypertension' di Sao Paolo, Brazil pada 15-19hb February 2004

(2) Memenangi tempat ketiga abstrak terbaik di 'First Scientific Meeting of the Malaysian Society of Hypertension, Kuala Lumpur, Oct 2003

(3) Sebuah kertas kerja hasil kajian ini akan dibentangkan pada 'Scientific Meeting, Malaysian Society of Pharmacology and Physiology' pada 17-18 Mei 2004

(ii) PENERBITAN SAINTIFIK

(1) Dependence of human forearm skin postocclusive reactive hyperemia on occlusion time. In press – Journal of Phamacological and Toxicological Methods (galley proof dikembarkan)

(2) Reproducible assessment of reactive hyperemia: Laser doppler fluximetry of the skin microcirculation. Submitted to British Journal of Pharmaoclogy

H. HUBUNGAN DENGAN PENYELIDIK LAIN

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(1) _____

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Sarjana	_____
	_____
Ph.D	**Tee Get Bee**

MAKLUMAT LAIN YANG BERKAITAN

Saya sedang memohon sebuah lagi geran untuk menyambung kajian dalam bidang ini – melihat kesan umur, jantina dan fasa menstrual pada fungsi salur darah manusia.

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15/08/2004
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Brief communication

Dependence of human forearm skin postocclusive reactive hyperemia on occlusion time

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Abstract

Introduction: Human postocclusive forearm skin reactive hyperemia is not only a potential means of identifying early signs of cardiovascular diseases, it can also be used in the assessment of local microvascular response to topically applied compounds on skin. The method is not fully characterized. In this study, we investigated the influence of occlusion time on postocclusive forearm skin reactive hyperemia using laser Doppler fluximetry (LDF). **Methods:** Twenty healthy male volunteers were studied on three separate days (at least 24 h apart) via a randomized design. Volunteers were studied in a supine position while fasted. Laser Doppler probes were placed on the volar surface of the antebrachium. In preliminary studies, 3 min of upper arm blood flow occlusion at suprasystolic pressure was found to be the upper limit of tolerability. Subsequently, volunteers were randomized to receive 1, 2, or 3 min occlusion on 3 different days. Skin blood flux was measured before, during, and after occlusion using LDF. The primary outcome calculated was maximal change in skin blood flux before and after occlusion, expressed in arbitrary units (AU). **Results:** Skin blood flux changes (mean ± S.E.M.) after 1, 2, and 3 min occlusion period were 15.39 ± 1.27 AU, 24.84 ± 1.62 AU, and 32.14 ± 1.73 AU, respectively. Using repeated-measures analysis of variance (ANOVA), significant difference ($P<.05$) in skin blood flux changes were revealed between these three occlusion durations, where 3 min occlusion produced significantly greater in skin blood flux occlusion change compared to 1 and 2 min occlusion. **Conclusion:** Three minutes of occlusion produces the greater postocclusive reactive hyperemia. It is recommended that studies using postocclusive forearm skin reactive hyperemia should occlude the forearm for at least 3 min.

Keywords: Laser Doppler; Methods; Microvascular; Reactive hyperemia; Skin

1. Introduction

The microvasculature, particularly the capillary, is the point where intimate contact and exchange between the cardiovascular system and tissues is completed. It is now a well-accepted concept that functional abnormalities of the microcirculation are the primary abnormalities in many cardiovascular diseases' pathogenesis. Reduced maximal vasodilator capacity in the microcirculation was reported in hypertensive participants at an early stage of hypertension (Taketshi et al., 1992). In young patients with Type 1 diabetes, changes in the skin microvasculature have been found to occur many years prior to the appearance of symptoms of microvascular disease in other organs (Khan, Elhadd, Greene, & Belch, 2000).

Postocclusive forearm skin reactive hyperemia is an increased skin blood flow to tissue that follows the release of a brief arterial occlusion. It corresponds to the reperfusion of the vascular beds and is characterized by a peak skin blood flux. Although the exact mechanism involving skin postischaemic hyperemia response is not yet fully understood, it is thought to be endothelium dependent (Beinder & Schlembach, 2001), involving both myogenic and metabolic factors (Banitt, Smits, Williams, Ganz, & Creager, 1996; Johnson, Burton, Henrich, & Henrich, 1976; Lambard & Duling, 1981; O'Leary, Dunlap, & Glover, 1994; Olesen, Clapham, & Davies, 1988). Both nitric oxide (Meredith et al., 1996) and prostaglandin

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(Binggeli et al., 2003) potentiate their vasodilatory functions in postocclusive forearm skin reactive hyperemia. The assessment of postocclusive forearm skin reactive hyperemia can provide important information about changes in microcirculation and is a sensitive indicator of atherogenesis (Carberry, Shepherd, & Johnson, 1992; Lusher & Noll, 1996). It indicates either reduced vasodilator bioavailability or enhanced vasoconstriction in response to hypoxia. Gidlof, Lewis, and Hammersen (1988) also noted that the degree of endothelial damage was inversely related to the degree of vasodilatation and reactive hyperemia.

Besides serving as an important indicator in pathogenesis of cardiovascular diseases, postocclusive forearm skin reactive hyperemia can be used in the assessment of systemic effects of drugs or local effects of topically applied compounds on skin microvascular blood flow. Injection of drugs locally into the skin can be facilitated by iontophoresis (Kubli, Feihl, & Waeber, 2001).

The measurement of postocclusive forearm skin reactive hyperemia flow can be performed noninvasively using laser Doppler fluximetry (LDF). LDF, also commonly known as laser Doppler flowmetry, provides real-time continuous measurements related to changes in microvascular blood perfusion.

The occlusion time used in postocclusive forearm skin reactive hyperemia studies varies, ranging from as short as 1 (Hassan, Otsuka, Shimose, Okada, & Togawa, 2000) to 5 min (Sieg-Dobrescu, Burnier, Hayoz, Brunner, & Waeber, 2001), and has not been standardized. Walmsley and Wiles (1990) have found that peak postischemic skin blood flow of dorsal foot continued to increase significantly with up to 10 min ischemia in nine participants. However, this study measured the blood flow in the skin of lower limb, which has different physiological perfusion properties than forearm skin (Tur, Tur, Maibach, & Guy, 1983). In a study investigating the effects of different wavelets used in improving LDF signals, investigators found that time to peak and peak flow decreased as the length of occlusion duration is reduced in five males and six females (Humeau, Saumet, & L'Huillier, 2002). This study was however not designed to investigate the effect of occlusion duration on postocclusive reactive hyperemia blood flow. It also studied both males and females. It has been demonstrated that skin blood flow is significantly affected by hormone levels (Bungum, Kvernebo, Qian, & Maltau, 1996).

Several animal studies have reported on the effects of occlusion times on reactive hyperemia (Johnson et al., 1976; Klabunde & Johnson, 1977; Lombard & Duling, 1981). Johnson et al. (1976) demonstrated that peak capillary flow of the sartorius muscles increased progressively after short occlusion (10–15 s) and only a moderate rise to 280% above control was seen following 120 s occlusion. Moreover, capillary reactive hyperemia peak velocity increased with increasing occlusion times (3.5–180 s) in the latissimus dorsi muscle of chicken (Klabunde & Johnson, 1977). A similar finding was also reported by Lombard and Duling (1981), who studied single arterioles in the hamster cheek pouch. The authors have shown that both upstream and downstream diameters of the arteriole increased during longer occlusion. These animal studies, however, demonstrated the effect of occlusion duration on postocclusive reactive hyperemia response only in a single component of microvasculature (e.g., capillary or arteriole only).

To address the outstanding issues, the objective of the present study was to investigate the effect of varying occlusion times on human postocclusive forearm skin reactive hyperemia by LDF using male volunteers.

2. Methods

2.1. Participants

Twenty nonsmoking male volunteers were recruited after medical examination and laboratory tests, including a full blood count and total plasma cholesterol levels. The inclusion criteria were as follows: (1) healthy male, 21–30 years old; (2) normotensive (blood pressure lower than 140/90 mm Hg); (3) total plasma cholesterol less than 6.5 mmol/l; and (4) normal full blood count profile. Individuals with bleeding disorders and abnormal platelet counts, on pharmacotherapy with known vascular effects (e.g., antiinflammatory or cardiovascular medications), those with Raynaud's phenomenon, previous arm surgery or known personal history of hypertension, diabetes, hypercholesterolemia, or other peripheral vascular diseases were excluded. None of the participants was obese (body mass index>30 kg/m^2) or had large arm circumference (>34 cm). Following explanation of the study protocol, its benefits and risks, participants signed a written informed consent. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethical Committee of Universiti Sains Malaysia.

2.2. Laser Doppler fluximetry

Measurement and changes in forearm skin blood flux were measured noninvasively using a DRT4 LDF (Moor Instruments, Axminster, UK). This device uses a weak laser light with 780- to 820-nm wavelengths, which is sent to the skin via a fiber-optic system. It permits real-time continuous measurement of microvascular perfusion. In the present study, this instrument was used together with DP1T-V2 skin laser probe (Moor Instruments), which was stably held by PH1-V2 probe holder (Moor Instruments). The laser light usually penetrates to a depth of about 1.5 mm (Fagrell, 1994). LDF uses the Doppler shift principle. Photons of laser light scattered in moving blood cells produce a Doppler shift on the reflected light. This reflected light is detected by a photodetector and the signal

is processed to determine the amount of the frequency shift. The recorded Doppler-shift signal corresponds to an average blood cell velocity obtained under an averaged angle. It also depends on the number of red blood cells in the sample volume. The resulting signal is therefore a product of the number of blood cells moving in the sample volume and the mean velocity of the moving blood cells. This outcome is generally termed as "flux" and expressed in arbitrary units (AU).

Flux = Blood cell volume fraction × Velocity

In most physiologic and clinical situations, LDF flux and volume blood flow have a good correlation. Study has demonstrated a good correlation between laser Doppler measurements and other methods of blood flow measurement, such as plethysmography (Johnson, 1984).

2.3. Protocol

All study sessions were conducted between 1200 and 1600 h in a quiet room at a constant ambient room temperature of 23 °C and a relative humidity of 70%. Prior to a study session, participants fasted overnight, refrained from any vigorous exercises for 12 h and abstained from alcohol- and caffeine-containing beverages for 48 h. Participants were familiarized with the instrumentation and study environment before testing. They were also asked to come for three different study days (24–72 h apart). On each study day, different occlusion duration (either 1, 2, or 3 min) was applied to each participant according to a random crossover schedule.

Upon arrival for a study session, participants were allowed 15 min of acclimatization in a comfortable supine position with the arms and hands kept stationary and with the right arm uncovered (Fig. 1). The right arm was supported by a cushion and was slightly elevated above heart level. Skin laser Doppler probes (Moor Instruments) were fixed separately on the volar surface of the right forearm. The location of the probes was recorded clearly to permit replication on subsequent study days.

After a 15-min rest in the supine position, blood pressure was taken followed by a baseline skin blood flux reading for 1 min. Forearm blood flow was then occluded using a pneumatic pressure cuff (Accoson, England) placed on the right upper arm (about 1–2 cm above the antecubital crease) and inflated at a suprasystolic pressure (200 mm Hg) for either 1, 2, or 3 min. The skin blood flux was then recorded for 2 min after the occlusion was released. Skin blood flux and temperature were measured continuously and simultaneously by LDF with the laser probes maintained at the same site for each participant.

As the variability of laser Doppler perfusion during rest is known to be substantial, the postocclusive forearm skin reactive hyperemia response was calculated (absolute change in skin blood flux = peak skin blood flux – minimum base-

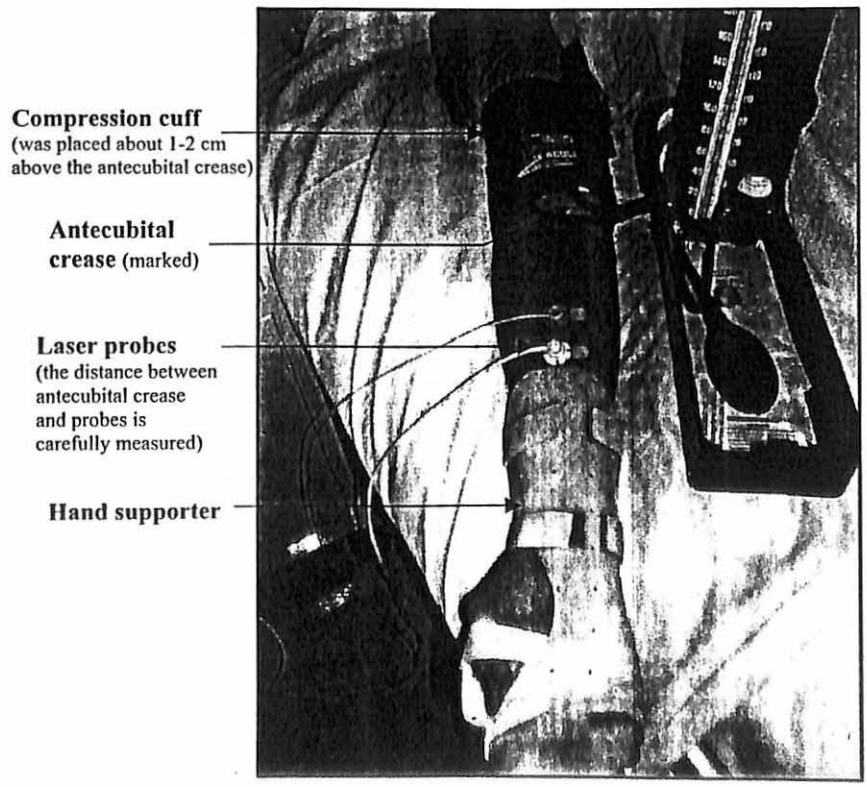

Fig. 1. Placement of laser Doppler probes, hand supporter, and compression cuff on a participant's right arm.

t1.1 Table 1
t1.2 Baseline characteristics of participants

t1.3 Parameter ($N=20$)	Mean ± S.E.M.
t1.4 Age (years)	24 ± 1
t1.5 Body mass index (kg/m^2)	21.66 ± 0.56
t1.6 Systolic blood pressure (mm Hg)	114.10 ± 1.86
t1.7 Diastolic blood pressure (mm Hg)	67.72 ± 1.41
t1.8 Pulse (beats/min)	68 ± 2
t1.9 Total plasma cholesterol (mmol/l)	4.82 ± 0.16
t1.10 Hemoglobin (g/dl)	14.53 ± 0.22
t1.11 Hematocrit (%)	43.41 ± 0.61
t1.12 Total white blood cells ($\times 10^9$/l)	7.10 ± 0.31
t1.13 Platelet ($\times 10^9$/l)	256.00 ± 9.53

line skin blood flux, in AU) to assess the reserve capacity of skin blood supply (Ubbink, Spincemaille, Reneman, & Jacobs, 1999).

2.4. Statistics

Statistical analysis was performed using the SPSS Software version 11.0 (SPSS, Chicago, IL, USA). A combination of the Shapiro–Wilks Test and normal distribution plots showed that data were approximately normally distributed. Therefore, repeated-measures analysis of variance (ANOVA) was used to analyze differences in skin blood flux changes between different occlusion durations. Significance was defined by $P<.05$. Values are expressed as mean ± S.E.M.

3. Results

Baseline data for the 20 participants in this study is shown in Table 1. The mean minimum baseline skin blood flux for 1, 2, and 3 min occlusion duration were 8.37 ± 0.36 AU, 8.74 ± 0.42 AU, and 8.76 ± 0.36 AU, respectively. No significant difference was observed between these baseline fluxes. There were substantial increases in skin blood flux after releasing of forearm occlusion. There was a twofold increase in skin blood flux after 1 min occlusion, a threefold increase was seen after 2 min occlusion, and there was a fourfold increase after 3 min occlusion. Mean skin blood flux changes after 1, 2, and 3 min occlusion are shown in Fig. 2. Using repeated-measures analysis, significant differences ($P<$ .001) in skin blood flux changes were seen between 1, 2, and 3 min occlusion duration (Fig. 2). Three minutes of occlusion produced significantly greater postocclusive change in skin blood flux compared to 1 and 2 min occlusion. Two minutes of occlusion also produced greater postocclusive changes compared to 1 min.

4. Discussion

The objective of the present study was to determine the effect of varying occlusion durations on postocclusive forearm skin reactive hyperemia. We have found that the

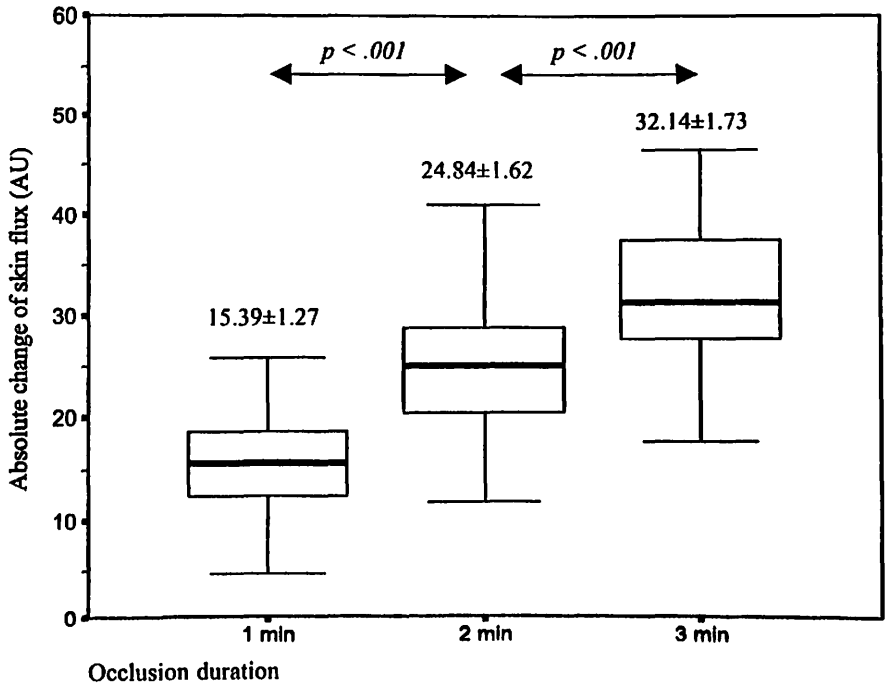

Fig. 2. Mean absolute change in skin blood flux postocclusion. For each category of occlusion duration, the box represents the interquartile range (between the 25th and 75th percentiles). The mean skin blood flux changes ($n=20$ for each occlusion duration) are stated above the boxes. The bars outside each box show the range of 95% of all value. Repeated-measures ANOVA showed significant difference in skin blood flux changes between 1 and 2 min, between 1 and 3 min, and between 2 and 3 min (all $P<.001$, general linear model, pairwise comparison).

mean skin blood flux change after 3 min occlusion was significantly greater compared to 1 and 2 min occlusion.

A wide range of occlusion durations has been applied in previous postocclusive skin reactive hyperemia studies using LDF or laser Doppler imaging. In a study by Hassan et al. (2000), 1 min of upper arm occlusion was used to visualize the effect of reactive hyperemia by measuring skin thermal inertia. More recent work by Vuilleumier et al. (2002) applied a 2-min upper arm occlusion in the Swedish female population. Studies using 3- (Kubli et al., 2001) and 5-min (Pellaton, Kubli, Feihl, & Waeber, 2002; Sieg-Dobrescu et al., 2001) durations were also reported to elicit skin reactive hyperemia. No reasons were given by the authors as to why these occlusion durations were chosen.

In the present study, we examined the postocclusive forearm skin reactive hyperemic response up to 3 min occlusion only. The limit of 3 min was set, based on the observation of a pilot study in which we found that occlusion durations longer than 3 min caused small but marked bruises at the compression sites in all participants ($n=5$). Longer durations also caused more discomfort to the participants. Three minutes was therefore deemed the upper limit for the study.

We demonstrated that changes in postocclusive skin reactive hyperemic response were not comparable between 1, 2, and 3 min occlusion duration. Three minutes of occlusion provided the most marked postocclusive vasodilatation and was well tolerated by all participants. In a separate unpublished study, we tested the reproducibility of postocclusive forearm skin reactive hyperemia response with 3 min occlusion in 18 healthy male volunteers. The result obtained showed that both intraday and interday variabilities were below 10% (Tee et al., unpublished data).

The fourfold increase in skin blood flux after 3 min occlusion was comparable with values reported by Kubli, Waeber, Dalle-Ave, and Feihl (2000). These investigators applied a 3-min occlusion at a pressure of 200 mm Hg in 16 participants. The skin blood flux response to reactive hyperemia was measured using laser Doppler imaging.

In the present study, the compression cuff was placed on the upper arm instead of putting it at the forearm. Upper arm cuff placement has been reported to elicit a greater flow stimulus due to more resistant vessel recruitment. A greater change in brachial artery diameter was seen after upper arm compression compared to the change produced by compression at the forearm (Coretti et al., 2002).

During skin blood flux measurement, several precautions were taken to increase accuracy. A constant room temperature and mean skin temperature were maintained throughout the study. Cold ambient temperature was avoided as this can cause the skin temperature and skin blood flux to drop (Liang, Su, & Lee, 2000). Precaution was also taken when attaching the probe so that it is held in contact with the skin without applying any unnecessary pressure, which would occlude the vessels under study. The location for attachments of probes was also carefully marked, measured and recorded to reduce site-to-site variations. During readings of skin blood flux, small shifts in the position of either the laser probes or the forearm can alter microvascular blood flow measurements substantially. Considerable attention was given in this study to maintain a constant position throughout each individual's study. A hand supporter was placed at the hand and forearm as to reduce involuntary hand movement during blood flow measurement (Fig. 1).

In the recent work done on 862 asymptomatic healthy women (Vuilleumier et al., 2002), the authors demonstrated a close relationship between postocclusive forearm skin reactive hyperemia measured by LDF and the weight of cardiovascular risk as assessed by the individual Framingham risk score. This finding suggests that postocclusive forearm skin reactive hyperemia response assessed by LDF might be a valuable noninvasive method to stratify cardiovascular risk. It may appear to be an easy method to assess microvascular health and in predicting macrovascular outcome. An impaired postocclusive forearm skin reactive hyperemia response could potentially identify a subpopulation at risk for cardiovascular complications.

In conclusion, this study showed that the magnitude of postocclusive skin reactive hyperemia in the forearm is dependent on the occlusion duration. Our data suggest that 3 min occlusion is the most suitable duration to use in studies of postocclusive forearm skin reactive hyperemia in our population. Occlusion shorter than 3 min produces submaximal hyperemia. Occlusion durations of more than 3 min may produce bigger responses but at the expense of volunteers' comfort.

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References

Banitt, P. F., Smits, P., Williams, G. B., Ganz, P., & Creager, M. A. (1996). Activation of ATP-sensitive potassium channels contributes to reactive hyperemia in humans. *American Journal of Physiology, 271*, H1594–H1598.

Beinder, E., & Schlembach, D. (2001). Skin flux during reactive hyperemia and local hyperthermia in patients with preeclampsia. *Obstetrics & Gynecology, 98*, 313–318.

Binggeli, C., Spieker, L. E., Corti, R., Sudano, I., Stojanovic, V., Hayoz, D., Lusher, T. F., & Noll, G. (2003). Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients. *Journal of the American College of Cardiology, 42*, 71–77.

Bungum, L., Kvernebo, K., Qian, P., & Maltau, J. M. (1996). Laser Doppler-recorded reactive hyperemia in the forearm skin during menstrual cycle. *British Journal of Obstetrics and Gynaecology, 103*(1), 70–75.

Carberry, P. A., Shepherd, A. M., & Johnson, J. M. (1992). Resting and maximal forearm skin blood flows are reduced in hypertension. *Hypertension, 20*, 349–355.

Coretti, M. C., Anderson, T. J., Benjamin, E. J., Celermajer, D., Charbonneau, F., Creager, M. A., Deanfield, J., Drexler, H., Gerhard-Herman,

M., Herrington, D., Vallance, P., Vita, J., & Vogel, R. (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilatation of the brachial artery. *Journal of the American College of Cardiology, 39*, 257–265.

Fagrell, B. (1994). Problems using laser Doppler on the skin in clinical practice. In A. N. Nicolaides (Ed.), *Laser Doppler* (pp. 49–54). London: Med-Orion Publishing.

Gidlof, A., Lewis, D., & Hammersen, F. (1988). The effect of prolonged total ischemia on the ultrastructure of human skeletal muscle capillaries: A morphometric analysis. *International Journal of Microcirculation, Clinical and Experimental, 7*, 257–265.

Hassan, M., Otsuka, K., Shimose, A., Okada, S., & Togawa, T. (2000). Imaging of thermal inertia to visualize reactive hyperemia in the forearm skin after arterial occlusion. *Proceedings of the 22nd IEEE/Engineering in Medicine and Biology Society, 22*, 1229–1231.

Humeau, A., Saumet, J., & L'Huillier, J. (2002). Use of wavelets to accurately determine parameters of laser Doppler reactive hyperemia. *Microvascular Research, 60*(2), 141–148.

Johnson, J. M. (1984). Laser Doppler measurement of skin blood flow: Comparison with plethysmography. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology, 56*, 798.

Johnson, P. C., Burton, K. S., Henrich, H., & Henrich, U. (1976). Effect of occlusion duration on reactive hyperemia in sartorius muscle capillaries. *American Journal of Physiology, 230*(3), 715–719.

Khan, F., Elhadd, T., Greene, S., & Belch, J. (2000). Impaired skin microvascular function in children, adolescents and young adults with type 1 diabetes. *Diabetes Care, 23*(2), 215–220.

Klabunde, R. E., & Johnson, P. C. (1977). Reactive hyperemia in capillaries of red and white skeletal muscle. *American Journal of Physiology, 232*(4), H411–H417.

Kubli, S., Feihl, F., & Waeber, B. (2001). Beta-blockade with nebivolol enhances the acetylcholine-induced cutaneous vasodilatation. *Clinical Pharmacology and Therapeutics, 69*, 238–244.

Kubli, S., Waeber, B., Dalle-Ave, A., & Feihl, F. (2000). Reproducibility of laser Doppler imaging of skin blood flow as a tool to assess endothelial function. *Journal of Cardiology and Pharmacology, 36*, 628–640.

Lambard, J. H., & Duling, B. R. (1981). Multiple mechanism of reactive hyperemia in arterioles of the hamster cheek pouch. *American Journal of Physiology, 241*, H748–H755.

Liang, T. C. M., Su, H. F., & Lee, N. Y. (2000). Skin temperature and skin blood flow affect bioelectric impedance study of female fat-free mass. *Medicine and Science in Sports and Exercise, 32*(1), 221–234.

Lombard, J. H., & Duling, B. R. (1981). Multiple mechanisms of reactive hyperemia in arterioles of the hamster cheek pouch. *American Journal of Physiology, 241*(5), H748–H755.

Lusher, T. F., & Noll, G. (1996). Endothelial function as an end-point in interventional trials: Concepts, methods and current data. *Journal of Hypertension, 14*, 111–119.

Meredith, I. T., Currie, K. E., Anderson, T. J., Roddy, M. A., Ganz, P., & Creager, M. A. (1996). Postischemic vasodilatation in human forearm is dependent on endothelium-derived nitric oxide. *American Journal of Physiology, 270*, H1435–H1440.

O'Leary, D. S., Dunlap, D. C., & Glover, K. W. (1994). Role of endothelium-derived relaxing factors in hindlamb reactive and active hyperemia in conscious dogs. *American Journal of Physiology, 266*, R1213–R1219.

Olesen, S. P., Clapham, D. E., & Davies, P. F. (1988). Hemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature, 331*, 168–170.

Pellaton, C., Kubli, S., Feihl, F., & Waeber, B. (2002). Blunted vasodilatory responses in the cutaneous microcirculation of cigarette smokers. *American Heart Journal, 144*(2), 269–274.

Sieg-Dobrescu, D., Burnier, M., Hayoz, D., Brunner, H., & Waeber, B. (2001). The return of increased blood pressure after discontinuation of antihypertensive treatment is associated with an impaired post-ischemic skin blood flow response. *Journal of Hypertension, 19*, 1387–1392.

Taketshi, A., Imaizumi, T., Ashihara, T., Yamamoto, K., Hoka, S., & Nakamura, M. (1992). Limited maximal vasodilator capacity of forearm resistance vessels in normotensive young men with familial predisposition to hypertension. *Circulation Research, 50*, 671–677.

Tur, E., Tur, M., Maibach, H. I., & Guy, G. H. (1983). Basal perfusion of cutaneous microcirculation: Measurements as a function of anatomic position. *Journal of Investigative Dermatology, 81*, 442–446.

Ubbink, D. T., Spincemaille, G. H., Reneman, R. S., & Jacobs, M. J. (1999). Prediction of imminent amputation in patients with non-reconstructible leg ischemia by means of microcirculatory investigations. *Journal of Vascular Surgery, 30*, 114–121.

Vuilleumier, P., Decosterd, D., Maillard, M., Constantin, C., Burnier, M., & Hayoz, D. (2002). Postischemic forearm skin reactive hyperaemia is related to cardiovascular risk factors in a healthy female population. *Journal of Hypertension, 20*, 1753–1757.

Walmsley, D., & Wiles, P. G. (1990). Reactive hyperemia in skin of the human foot measured by laser Doppler flowmetry: Effects of duration of ischemia and local heating. *International Journal of Microcirculation, Clinical and Experimental, 9*(4), 345–355.

Study objectives

To optimise a method for assessing microvascular endothelial function using reactive hyperemia and laser doppler flowmetry

To establish the reproducibility of the above method in assessing endothelial function

Hypothesis:

Reactive hyperemia of dermal microcirculation as measured by laser doppler flowmetry gives highly reproducible results.

General Methodology

This project is divided into 2 studies

1. Study 1: Aimed to determine the optimum occlusion duration for reactive hyperemia at forearm

2. Stduy 2: Aimed to determine the interday and intraday reproducibility of laser doppler flowmetry for assessing microvascular endothelial function

The detailed introduction, methods, results, conclusion and discussion for each study is given.

Abstract

Method Optimisation and Validation Study on use of Laser Doppler Flowmetry to Assess Microvascular Endothelial Function

Introduction Human postocclusive forearm skin reactive hyperemia method is a potential means of identifying early signs of cardiovascular diseases. In this study, we investigated the effect of varying occlusion times in assessing postocclusive forearm skin reactive hyperemia using laser Doppler fluximetry (LDF). We also determined the intraday and interday reproducibility of this method.

Methods Twenty healthy male volunteers were studied on three separate days (at least 24 hours apart) via a randomized design. Volunteers were studied in a supine position while fasted. Laser Doppler probes were placed on the volar surface of the antebrachium. Upper arm blood flow occlusion at suprasystolic pressure was applied and duration of occlusion on each day was randomized to 1, 2 or 3 minutes. Skin blood flux was measured before, during and after occlusion using LDF. The primary outcome calculated was maximal change in skin blood flux before and after occlusion, expressed in arbitrary unit (AU). To address intraday and interday reproducibility, skin reactive hyperemia was performed twice within each study day for 2 study days. Intraday assessments were separated by 60-90 minutes; while the interday assessments were 24-72 hours apart.

Results Skin blood flux changes (mean±SEM) after 1, 2 and 3 minutes occlusion period were 15.39±1.27AU, 24.84±1.62AU and 32.14±1.73AU respectively. Using repeated measures analysis of variance, significant difference ($p<0.05$) in skin blood flux changes were revealed between these three occlusion durations, where 3 minutes occlusion produced significantly greater in skin blood flux occlusion change compared to 1 and 2 minutes. The intraday and interday coefficient of variation of skin blood flux measurement was 4.773% and 6.504%, respectively. Intraclass correlation coefficient for intraday was 0.9718 and for interday was 0.9281. Using paired t-test (95% CI), there was no significant difference observed within intraday and interday measurements.

Conclusion Three minutes occlusion produces the maximum postocclusive reactive hyperemia measured by laser Doppler fluximetry. It is a well-tolerated and highly reproducible method. It is recommended that studies using postocclusive forearm skin reactive hyperemia should occlude the forearm for at least 3 minutes.

STUDY I

METHOD OPTIMISATION ON USE OF LASER DOPPLER FLOWMETERY TO ASSESS MICROVASCULAR ENDOTHELIAL FUNCTION

Abstract

Introduction Human postocclusive forearm skin reactive hyperemia is not only a potential means of identifying early signs of cardiovascular diseases, it also can be used in the assessment of local microvascular response to topically applied compounds on skin. The method is not fully characterised. In this study, we investigated the influence of occlusion time on postocclusive forearm skin reactive hyperemia using laser Doppler fluximetry (LDF).

Methods Twenty healthy male volunteers were studied on three separate days (at least 24 hours apart) via a randomized design. Volunteers were studied in a supine position while fasted. Laser Doppler probes were placed on the volar surface of the antebrachium. In preliminary studies, 3 minutes of upper arm blood flow occlusion at suprasystolic pressure was found to be the upper limit of tolerability. Subsequently, volunteers were randomized to receive 1, 2 or 3 minutes occlusion on 3 different days. Skin blood flux was measured before, during and after occlusion using LDF. The primary outcome calculated was maximal change in skin blood flux before and after occlusion, expressed in arbitrary units (AU).

Results Skin blood flux changes (mean±SEM) after 1, 2 and 3 minutes occlusion period were 15.39±1.27AU, 24.84±1.62AU and 32.14±1.73AU respectively. Using repeated measures analysis of variance, significant difference ($p<0.05$) in skin blood flux changes were revealed between these three occlusion durations, where 3 minutes occlusion produced significantly greater in skin blood flux occlusion change compared to 1 and 2 minutes.

Conclusion Three minutes occlusion produces the greater postocclusive reactive hyperemia. It is recommended that studies using postocclusive forearm skin reactive hyperemia should occlude the forearm for at least 3 minutes.

1. Introduction

The microvasculature, particularly the capillary, is the point where intimate contact and exchange between the cardiovascular system and tissues is completed. It is now a well-accepted concept that functional abnormalities of the microcirculation are the primary abnormalities in many cardiovascular diseases' pathogenesis. Reduced maximal vasodilator capacity in the microcirculation was reported in hypertensive subjects at an early stage of hypertension (Taketshi et al., 1992). In young patients with type 1 diabetes, changes in the skin microvasculature have been found to occur many years prior to the appearance of symptoms of microvascular disease in other organ (Khan, Elhadd, Greene, & Belch, 2000).

Postocclusive forearm skin reactive hyperemia is an increased skin blood flow to tissue that follows the release of a brief arterial occlusion. It corresponds to the reperfusion of the vascular beds and is characterized by a peak skin blood flux. Although the exact mechanism involved skin postischaemic hyperemia response is not yet fully understood, it is thought to be endothelium dependent (Beinder & Schlembach, 2001), involving both myogenic and metabolic factors (Banitt, Smits, Williams, Ganz, & Creager, 1996; Johnson, Burton, Henrich, & Henrich, 1976a; Lambard & Duling, 1981; O'Leary, Dunlap, & Glover, 1994; Olesen, Clapham, & Davies, 1988). Both nitric oxide (Meredith et al., 1996) and prostaglandin (Binggeli et al., 2003) potentiate their vasodilatory functions in postocclusive forearm skin reactive hyperemia. The assessment of postocclusive forearm skin reactive hyperemia can provide important information about changes in microcirculation and is a sensitive indicator of atherogenesis (Carberry, Shepherd, & Johnson, 1992; Lusher & Noll, 1996). It indicates either reduced in vasodilator bioavailability or enhanced vasoconstriction in response to hypoxia. Gidlof (Gidlof, Lewis, & Hammersen, 1988) also noted that the degree of endothelial damage was inversely related to the degree of vasodilatation and reactive hyperemia.

Besides serving as an important indicator in pathogenesis of cardiovascular diseases, postocclusive forearm skin reactive hyperemia can be used in the assessment of systemic effects of drugs or local effects of topically applied compounds on skin microvascular blood flow. Injection of drugs into the skin locally can be facilitated by iontophoresis (Kubli, Feihl, & Waeber, 2001).

The measurement of postocclusive forearm skin reactive hyperemia flow can be performed non-invasively using laser Doppler fluximetry. Laser Doppler fluximetry, also commonly known as laser Doppler flowmetry, provides real-time continuous measurements related to changes in microvascular blood perfusion.

The occlusion time used in postocclusive forearm skin reactive hyperemia studies varies, ranging from as short as 1 minute (Hassan, Otsuka, Shimose, Okada, & Togawa, 2000) to 5 minutes (Sieg-Dobrescu, Burnier, Hayoz, Brunner, & Waeber, 2001), and has not been standardized. Walmsley and colleagues have found that peak post-ischemic skin blood flow of dorsal foot continued to increase significantly with up to 10 minutes ischemia in 9 subjects (Walmsley & Wiles, 1990). However, this study measured the blood flow in the skin of lower limb, which has different physiological perfusion properties than forearm skin (Tur, Tur, Maibach, & Guy, 1983). In a study investigating the effects of different wavelets used in improving laser Doppler fluximetry signals, investigators found that time to peak and peak flow decreased as the length of occlusion duration is reduced in 5 males and 6 females (Humeau, Saumet, & L'Huillier, 2002). This study was however not designed to investigate the effect of occlusion duration on postocclusive reactive hyperemia blood flow. It also studied both males and females. It has been demonstrated that skin blood flow is significantly affected by hormone levels (Bungum, Kvernebo, Qian, & Maltau, 1996).

Several animal studies have reported on the effects of occlusion times on reactive hyperemia (Johnson, Burton, Henrich, & Henrich, 1976b; Klabunde & Johnson, 1977; Lombard & Duling, 1981). Johnson and colleagues demonstrated

that peak capillary flow of the sartorius muscles increased progressively after short occlusion (10-15 seconds) and only a moderate rise to 280% above control was seen following 120 seconds occlusion (Johnson, Burton, Henrich, & Henrich, 1976b). Also, capillary reactive hyperemia peak velocity increased with increasing occlusion times (3.5–180 seconds) in the latissimus dorsi muscle of chicken (Klabunde & Johnson, 1977). A similar finding was also reported by Lambard, who studied single arterioles in the hamster cheek pouch (Lombard & Duling, 1981). The authors have shown that both upstream and downstream diameters of the arteriole increased during longer occlusion. These animal studies, however, demonstrated the effect of occlusion duration on postocclusive reactive hyperemia response only in a single component of microvascular (e.g. capillary or arteriole only).

To address the outstanding issues, the objective of present study was to investigate effect of varying occlusion times on human postocclusive forearm skin reactive hyperemia using laser Doppler fluximetry using male volunteers.

2. Methods

2.1. Subjects

Twenty non-smoking male volunteers were recruited after medical examination and laboratory tests, including a full blood count and total plasma cholesterol levels. The inclusion criteria were: (1) Healthy male, aged 21- 30 years old; (2) Normotensive (blood pressure lower than 140/90mmHg); (3) Total plasma cholesterol less than 6.5mmol/l; (4) Normal full blood count profile. Individuals with bleeding disorders and abnormal platelet count, on pharmacotherapy with known vascular effects (e.g. anti-inflammatory or cardiovascular medications), those with Raynaud's phenomenon, previous arm surgery or known personal history of hypertension, diabetes, hypercholesterolemia or other peripheral vascular diseases were excluded. None of the participant was obese (body mass index >30kg/m^2) or had large arm circumference (>34cm). Following explanation of the study protocol, its benefits and risks, subjects signed a written informed consent. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethical Committee of Universiti Sains Malaysia.

2.2. Laser Doppler fluximetry

Measurement and changes in forearm skin blood flux were measured non-invasively using a DRT4 laser Doppler fluximetry (Moor Instruments, Axminster, U.K.). This device uses a weak laser light with 780-820nm wavelengths, which is sent to the skin via a fiber optic system. It permits real-time continuous measurement of microvascular perfusion. In the present study, this instrument was used together with DP1T-V2 skin laser probe (Moor Instruments, Axminster, U.K.), which was stably held by PH1-V2 probe holder (Moor Instruments, Axminster, U.K.). The laser light usually penetrates to a depth of about 1.5mm (Fagrell, 1994). Laser Doppler fluximetry uses Doppler shift principle. Photons of laser light scattered in moving blood cells produce a Doppler shift on the reflected light. This reflected light is detected by a photodetector and the signal is processed to determine the amount of the frequency shift. The recorded Doppler-shift signal corresponds to an average blood cell velocity obtained under an averaged angle. It also depends on the number of red blood cells in the sample volume. The resulting signal is therefore a product of the number of blood cells moving in the sample volume and the mean velocity of the moving blood cells. This outcome is generally termed as "flux" and expressed in Arbitrary Units (AU).

Flux = Blood cell volume fraction x velocity

In most physiologic and clinical situations, laser Doppler fluximetry flux and volume blood flow have a good correlation. Study has demonstrated a good correlation between laser Doppler measurements and other method of blood flow measurement, such as plethysmography (Johnson, 1984).

2.3. Protocol

All study sessions were conducted between 1200 and 1600 in a quite room at a constant ambient room temperature of 23°C and a relative humidity of 70%. Prior to a study session, subjects fasted overnight, refrained from any vigorous exercises for 12 hours and abstained from alcohol and caffeine-containing beverages for 48 hours. Subjects were familiarized with the instrumentation and study environment before testing. They were also asked to come for 3 different study days (24-72 hours apart). On each study day, different occlusion duration (either 1, 2 or 3 minutes) was applied to each subject according to a random cross-over schedule.

On arrival for a study session, subjects were allowed 15 minutes of acclimatization in a comfortable supine position with the arms and hands were kept stationary and with the right arm uncovered (Figure 1). The right arm was supported by a cushion and slightly elevated above heart level. Skin laser Doppler probes (Moor Instruments, Axminster, U.K.) were fixed separately on the volar surface of the right forearm. The location of the probes was recorded clearly to permit replication on subsequent study days.

After 15 minutes resting supine, blood pressure was taken followed by a baseline skin blood flux reading for one minute. Forearm blood flow was then occluded using a pneumatic pressure cuff (Accoson, England) placed on the right upper arm (about 1-2cm above the antecubital crease) and inflated at a suprasystolic pressure (200mmHg) for either 1, 2 or 3 minutes. The skin blood flux was then recorded for 2 minutes after the occlusion was released. Skin blood flux and temperature were measured continuously and simultaneously by laser Doppler fluximetry with the laser probes maintained at the same site for each subject.

As the variability of laser Doppler perfusion during resting is known to be substantial, the postocclusive forearm skin reactive hyperemia response was calculated (Absolute change in skin blood flux = peak skin blood flux minus the

minimum baseline skin blood flux, in AU) in order to assess the reserve capacity of skin blood supply (Ubbink, Spincemaille, Reneman, & Jacobs, 1999).

2.4. Statistics

Statistical analysis was performed using the SPSS Software version 11.0 (SPSS Inc., Chicago, IL, U.S.A.). A combination of the Shapiro-Wilks test and normal distribution plots showed that data were approximately normally distributed. Therefore, repeated measures analysis of variance (ANOVA) was used to analyze differences in skin blood flux changes between different occlusion durations. Significance was defined by $p<0.05$. Values are expressed as mean±standard error of mean (SEM).

3. Results

Baseline data for the 20 subjects in this study is shown in Table 1. The mean minimum baseline skin blood flux for 1, 2 and 3 minutes occlusion duration were 8.37±0.36AU, 8.74±0.42AU and 8.76±0.36AU respectively. No significant difference was observed between these baseline fluxes. There were substantial increases in skin blood flux after releasing of forearm occlusion. There was a 2-fold increase in skin blood flux after 1 minute occlusion, a 3-fold increase was seen after 2 minutes occlusion and there was a 4-fold increase after 3 minutes of occlusion. Mean skin blood flux changes after 1, 2 and 3 minutes occlusion are shown in Figure 2. Using repeated measures analysis, significant differences ($p<0.001$) in skin blood flux changes were seen between 1, 2 and 3 minutes occlusion duration (Figure 2). Three minutes occlusion produced significantly greater postocclusive change in skin blood flux compared to 1 and 2 minutes. Two minutes occlusion also produced greater postocclusive changes compared to 1 minute.

4. Discussion

The objective of the present study was to determine the effect of varying occlusion durations on postocclusive forearm skin reactive hyperemia. We have found that the mean skin blood flux change after 3 minutes occlusion was significantly greater compared to 1 and 2 minutes of occlusion.

A wide range of occlusion durations have been applied in previous postocclusive skin reactive hyperemia studies using laser Doppler fluximetry or laser Doppler imaging. In a study by Hassan *et al.*, 1 minute of upper arm occlusion was used to visualize the effect of reactive hyperemia by measuring skin thermal inertia (Hassan et al., 2000). More recently work by Vuilleumier applied a 2 minutes upper arm occlusion in the Swedish female population (Vuilleumier et al., 2002). Studies using 3 minutes (Kubli et al., 2001) and 5 minutes (Pellaton, Kubli, Feihl, & Waeber, 2002; Sieg-Dobrescu et al., 2001) durations were also reported to elicit skin reactive hyperemia. No reasons were given by the authors as to why these occlusion durations were chosen.

In the present study, we examined the postocclusive forearm skin reactive hyperemic response up to 3 minutes of occlusion only. The limit of 3 minutes was set based on the observation of a pilot study, in which we found that occlusion durations longer than 3 minutes caused small but marked bruises at the compression sites in all subjects (n=5). Longer durations also caused more discomfort to the subjects. Three min was therefore deemed the upper limit for the study.

We demonstrated that changes in postocclusive skin reactive hyperemic response were not comparable between 1, 2 and 3 minutes occlusion duration. Three minutes occlusion provided the most marked postocclusive vasodilatation and was well tolerated by all subjects. In a separate unpublished study, we tested the reproducibility of postocclusive forearm skin reactive hyperemia response with 3

minutes occlusion in 18 healthy male volunteers. The result obtained showed that both intraday and interday variability were below 10% (Tee *et al.*, unpublished data).

The 4-fold increase in skin blood flux after 3 minutes of occlusion was comparable with values reported by Kubli and colleagues (Kubli, Waeber, Dalle-Ave, & Feihl, 2000). These investigators applied a 3 minutes occlusion at a pressure of 200mmHg in 16 subjects. The skin blood flux response to reactive hyperemia was measured using laser Doppler imaging.

In the present study, the compression cuff was placed on the upper arm instead of putting it at the forearm. Upper arm cuff placement has been reported to elicit a greater flow stimulus due to more resistance vessel recruitment. A greater change in brachial artery diameter was seen after upper arm compression compared to the change produced by compression at forearm (Coretti *et al.*, 2002).

During skin blood flux measurement, several precautions were taken to increase accuracy. A constant room temperature and mean skin temperature were maintained throughout the study. Cold ambient temperature was avoided as this can cause the skin temperature and skin blood flux to drop (Liang, Su, & Lee, 2000). Precaution was also taken when attaching the probe so that it is held in contact with the skin without applying any unnecessary pressure, which would occlude the vessels under study. The location for attachments of probes was also carefully marked, measured and recorded to reduce site-to-site variations. During readings of skin blood flux, small shifts in the position of either the laser probes or the forearm can alter microvascular blood flow measurements substantially. Considerable attention was given in this study to maintain a constant position throughout each individual's study. A hand supporter was placed at the hand and forearm as to reduce involuntary hand movement during blood flow measurement (Figure 1).

In the recent work done on 862 asymptomatic healthy women (Vuilleumier *et al.*, 2002), the authors demonstrated a close relationship between postocclusive forearm skin reactive hyperemia measured by laser Doppler fluximetry and the

weight of cardiovascular risk as assessed by the individual Framingham risk score. This finding suggesting that postocclusive forearm skin reactive hyperemia response assessed by laser Doppler fluximetry might be a valuable non-invasive method to stratify cardiovascular risk. It may appear to be an easy method to assess microvascular health and in predicting macrovascular outcome. An impaired postocclusive forearm skin reactive hyperemia response could potentially identify a subpopulation at risk for cardiovascular complications.

In conclusion, this study showed that the magnitude of postocclusive skin reactive hyperemia in the forearm is dependent on the occlusion duration. Our data suggest that 3 minutes occlusion is the most suitable duration to use in studies of postocclusive forearm skin reactive hyperemia, in our population. Occlusion shorter than 3 minutes produces submaximal hyperemia. Occlusion durations of more than 3 minutes may produce bigger responses but at the expense of volunteers comfort.

References

Banitt, P. F., Smits, P., Williams, G. B., Ganz, P., & Creager, M. A. (1996). Activation of ATP-sensitive potassium channels contributes to reactive hyperemia in humans. *Am J Physiol, 271*, H1594-H1598.

Beinder, E., & Schlembach, D. (2001). Skin flux during reactive hyperemia and local hyperthermia in patients with preeclampsia. *Obstet Gynecol, 98*, 313-318.

Binggeli, C., Spieker, L. E., Corti, R., Sudano, I., Stojanovic, V., Hayoz, D., Lusher, T. F., & Noll, G. (2003). Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients. *J Am Coll Cardiol, 42*, 71-77.

Bungum, L., Kvernebo, K., Qian, P., & Maltau, J. M. (1996). Laser doppler-recorded reactive hyperemia in the forearm skin during menstrual cycle. *Br J Obstet Gynaecol, 103 (1)*, 70-75.

Carberry, P. A., Shepherd, A. M., & Johnson, J. M. (1992). Resting and maximal forearm skin blood flows are reduced in hypertension. *Hypertension, 20*, 349-355.

Coretti, M. C., Anderson, T. J., Benjamin, E. J., Celermajer, D., Charbonneau, F., Creager, M. A., Deanfield, J., Drexler, H., Gerhard-Herman, M., Herrington, D., Vallance, P., Vita, J., & Vogel, R. (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilatation of the brachial artery. *J Am Coll Cardiol, 39*, 257-265.

Fagrell, B. (1994). Problems using laser Doppler on the skin in clinical practice. In A. N. Nicolaides (Ed.), *Laser Doppler* (pp. 49-54). London, Los Angeles, Nicosia: Med-Orion Publishing Company.

Gidlof, A., Lewis, D., & Hammersen, F. (1988). The effect of prolonged total ischemia on the ultrastructure of human skeletal muscle capillaries: a morphometric analysis. *Int. J. Microcirc.: Clin. Exp., 7*, 257-265.

Hassan, M., Otsuka, K., Shimose, A., Okada, S., & Togawa, T. (2000). Imaging of thermal inertia to visualize reactive hyperemia in the forearm skin after arterial occlusion. *Proc. of 22nd IEEE EMBS, 22*, 1229-1231.

Humeau, A., Saumet, J., & L'Huillier, J. (2002). Use of wavelets to accurately determine parameters of laser Doppler reactive hyperemia. *Microvasc Res, 60 (2)*, 141-148.

Johnson, J. M. (1984). Laser Doppler measurement of skin blood flow: comparison with plethysmography. *J Appl Physiol Respirat Environ Exercise Physiol, 56*, 798.

Johnson, P. C., Burton, K. S., Henrich, H., & Henrich, U. (1976b). Effect of occlusion duration on reactive hyperemia in sartorius muscle capillaries. *Am J Physiol, 230 (3)*, 715-719.

Khan, F., Elhadd, T., Greene, S., & Belch, J. (2000). Impaired skin microvascular function in children, adolescents and young adults with type 1 diabetes. *Diabetes Care, 23*(2), 215-220.

Klabunde, R. E., & Johnson, P. C. (1977). Reactive hyperemia in capillaries of red and white skeletal muscle. *Am J Physiol, 232 (4)*, H411-H417.

Kubli, S., Feihl, F., & Waeber, B. (2001). Beta-blockade with nebivolol enhances the acetylcholine-induced cutaneous vasodilatation. *Clin Pharmacol Ther, 69*, 238-244.

Kubli, S., Waeber, B., Dalle-Ave, A., & Feihl, F. (2000). Reproducibility of laser Doppler imaging of skin blood flow as a tool to assess endothelial function. *J of Cardiol Pharmacol, 36*, 640-628.

Lambard, J. H., & Duling, B. R. (1981). Multiple mechanism of reactive hyperemia in arterioles of the hamster cheek pouch. *Am J Physiol, 241*, H748-H755.

Liang, T. C. M., Su, H. F., & Lee, N. Y. (2000). Skin temperature and skin blood flow affect bioelectric impedance study of female fat-free mass. *Med Sci Sports Exerc, 32*(1), 221-234.

Lombard, J. H., & Duling, B. R. (1981). Multiple mechanisms of reactive hyperemia in arterioles of the hamster cheek pouch. *Am J Physiol, 241 (5)*, H748-H755.

Lusher, T. F., & Noll, G. (1996). Endothelial function as an end-point in interventional trials: concepts, methods and current data. *J Hypertens, 14*, 111-119.

Meredith, I. T., Currie, K. E., Anderson, T. J., Roddy, M. A., Ganz, P., & Creager, M. A. (1996). Postischemic vasodilatation in human forearm is dependent on endothelium-derived nitric oxide. *Am J Physiol, 270*, H1435-1440.

O'Leary, D. S., Dunlap, D. C., & Glover, K. W. (1994). Role of endothelium-derived relaxing factors in hindlamb reactive and active hyperemia in conscious dogs. *Am J Physiol, 266*, R1213-1219.

Olesen, S. P., Clapham, D. E., & Davies, P. F. (1988). Hemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature, 331*(168-170).

Pellaton, C., Kubli, S., Feihl, F., & Waeber, B. (2002). Blunted vasodilatory responses in the cutaneous microcirculation of cigarette smokers. *Am Heart J, 144*(2), 269-274.

Sieg-Dobrescu, D., Burnier, M., Hayoz, D., Brunner, H., & Waeber, B. (2001). The return of increased blood pressure after discontinuation of antihypertensive treatment is associated with an impaired post-ischemic skin blood flow response. *J Hypertens., 19*, 1387-1392.

Taketshi, A., Imaizumi, T., Ashihara, T., Yamamoto, K., Hoka, S., & Nakamura, M. (1992). Limited maximal vasodilator capacity of forearm resistance vessels in normotensive young men with familial predisposition to hypertension. *Circ Res, 50*, 671-677.

Tur, E., Tur, M., Maibach, H. I., & Guy, G. H. (1983). Basal perfusion of cutaneous microcirculation: measurements as a function of anatomic position. *J Invest Dermatol, 81*, 442-446.

Ubbink, D. T., Spincemaille, G. H., Reneman, R. S., & Jacobs, M. J. (1999). Prediction of imminent amputation in patients with non-reconstructible leg ischemia by means of microcirculatory investigations. *J Vasc Surg, 30*, 114-121.

Vuilleumier, P., Decosterd, D., Maillard, M., Constantin, C., Burnier, M., & Hayoz, D. (2002). Postischemic forearm skin reactive hyperaemia is related to cardovascular risk factors in a healthy female population. *J Hypertens, 20*, 1753-1757.

Walmsley, D., & Wiles, P. G. (1990). Reactive hyperemia in skin of the human foot measured by laser Doppler flowmetry: effects of duration of ischemia and local heating. *Int J Microcirculation: Clin Exp, 9 (4)*, 345-355.

Tables

Table 1

Baseline characteristics of subjects

PARAMETER (N=20)	MEAN ± SEM
Age (years)	24 ± 1
Body mass index (kg/m^2)	21.66 ± 0.56
Systolic blood pressure (mmHg)	114.10 ± 1.86
Diastolic blood pressure (mmHg)	67.72 ± 1.41
Pulse (beats/min)	68 ± 2
Total plasma cholesterol (mmol/l)	4.82 ± 0.16
Hemoglobin (g/dl)	14.53 ± 0.22
Hematocrit (%)	43.41 ± 0.61
Total white blood cells ($x10^9/l$)	7.10 ± 0.31
Platelet ($x10^9/l$)	256.00 ± 9.53

Figures

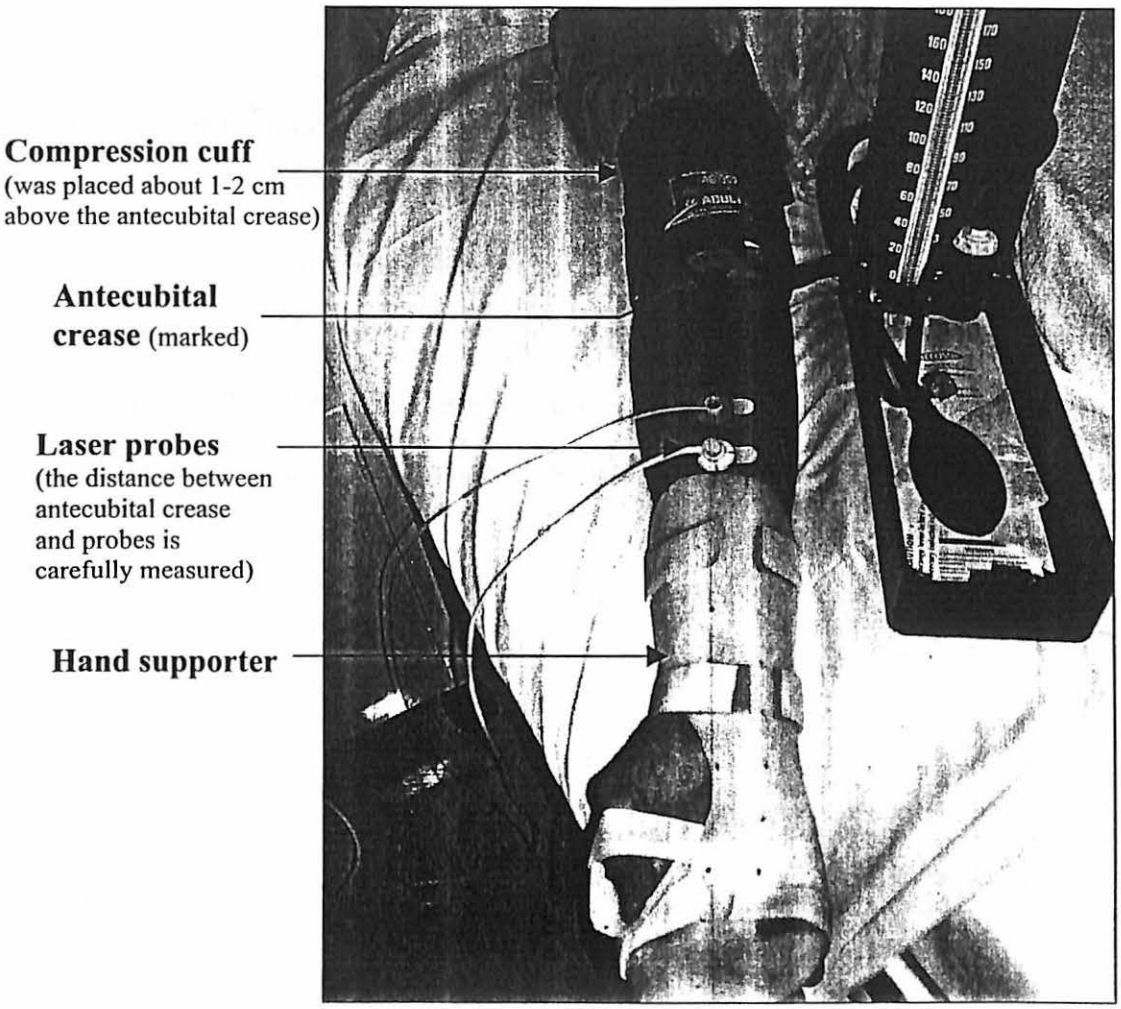

Fig. 1. Placement of laser Doppler probes, hand supporter and compression cuff on a subject's right arm.

Figure legends

Legend to Fig. 2.

For each category of occlusion duration, the box represents the interquartile range (between the 25th and 75th percentiles). The mean skin blood flux changes (n=20 for each occlusion duration) are stated above the boxes. The bars outside each box show the range of 95% of all value. Repeated measures ANOVA showed significant difference in skin blood flux changes between 1 min *vs.* 2 min, 1 min *vs.* 3 min and 2 min *vs.* 3 min (all $p<0.001$, general linear model, pairwise comparison).

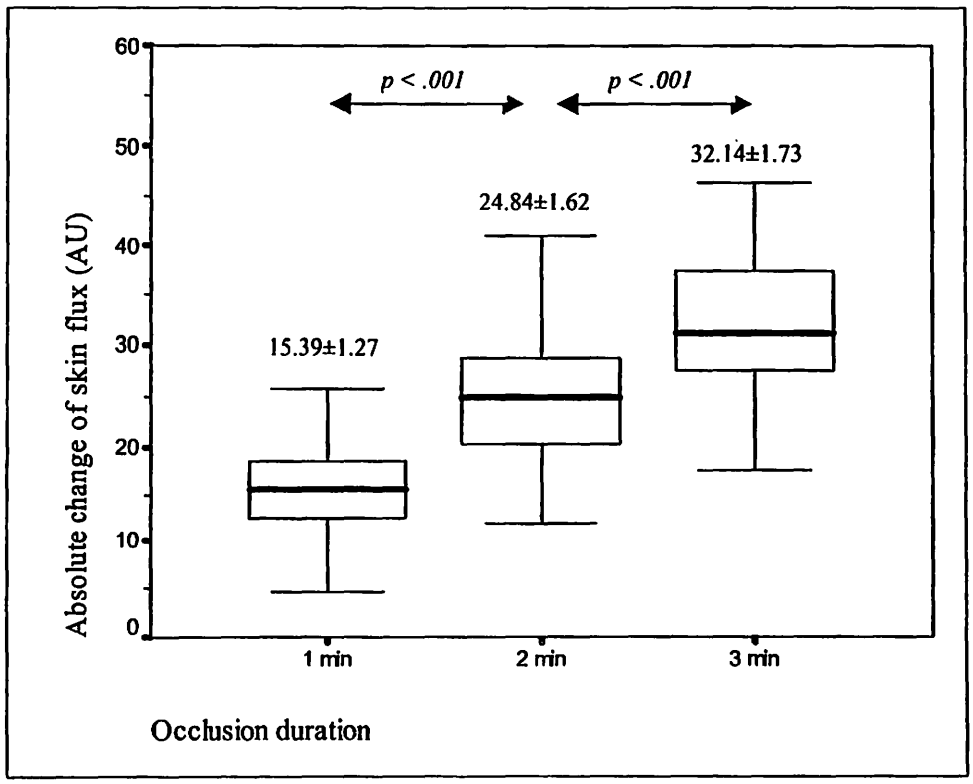

Fig. 2. Mean absolute change in skin blood flux postocclusion

STUDY II

REPRODUCIBLE ASSESSMENT OF POSTOCCLUSIVE FOREARM SKIN REACTIVE HYPEREMIA: LASER DOPPLER FLUXIMETRY OF THE SKIN MICROCIRCULATION

Reproducible Assessment of Postocclusive Forearm Skin Reactive Hyperemia: Laser Doppler Fluximetry of the Skin Microcirculation

ABSTRACT

Background Postocclusive skin reactive hyperemia is a useful indicator in assessment of functional reactivity of vascular bed. The aim of this study is to determine the intraday and interday variability of laser Doppler fluximetry for the assessment of postocclusive forearm skin reactive hyperemia response in humans.

Methods Eighteen young healthy male volunteers were recruited and studied in a supine position while fasted. Forearm blood flow was occluded at suprasystolic pressure for 3 minutes. Continuous skin blood flux measurement was made before, during and after occlusion using laser Doppler fluximetry. Absolute change in skin blood flux before and after occlusion was calculated. To address intraday and interday reproducibility, skin reactive hyperemia was performed twice within each study day for 2 study days. Intraday assessments were separated by 60-90 minutes; while the interday assessments were 24-72 hours apart. Results were expressed as mean+/-SEM and 95% confidence interval for mean differences.

Results Baseline age and body mass index were 23.78+/-0.27 years and 21.72+/-0.59 kg/m^2. The intraday and interday coefficient of variation of skin blood flux measurement was 4.773% and 6.504%, respectively. Intraclass correlation coefficient for intraday was 0.9718 and for interday was 0.9281. Using paired t-test, there was no significant difference observed within intraday and interday measurements.

Conclusions This study demonstrated that the technique of laser Doppler fluximetry coupled with skin reactive hyperemia with 3-minutes occlusion duration is a well-tolerated and highly reproducible method.

Introduction

Postocclusive skin reactive hyperemia is a protective physiological vasodilatation response that occurs to ensure adequate restoration of skin blood flow following a brief circulation arrest. The peak flow seen during reactive hyperemia is corresponding to the reperfusion capability of vascular beds. It is currently being investigated as a useful indicator of unaltered functional reactivity of vascular beds. Though the exact mechanism involved postocclusive skin reactive hyperemia is not yet clearly understood, it is though to result from interplay between myogenic and local vasoactive substances [1-6].

The assessment of postocclusive skin reactive hyperaemia provides important information about changes in microcirculation and is a sensitive indicator of atherogenesis [7, 8]. It indicates either reduced in vasodilator bioavailability or enhanced vasoconstriction in response to hypoxia. Besides serving as an important indicator in pathogenesis of cardiovascular diseases [9-11], postocclusive skin reactive hyperemia method can be used in the assessment of systemic effects of drug or local effects of topically applied compounds on microvascular functional reactivity.

The measurement of postocclusive forearm skin reactive hyperemia flow can be performed non-invasively using laser Doppler fluximetry. Laser Doppler fluximetry, also commonly known as laser Doppler flowmetry, provides real-time continuous measurements related to changes in microvascular blood perfusion. It utilizes the Doppler shift of laser light as the information carrier. Yet, this laser-Doppler skin reactive hyperemia method has not been optimized and standardized. The previous literatures reveal a wide diversion of occlusion duration used. Also, the convincing studies concerning reproducibility of the technique are scarce. In our previous study, we have shown that postocclusive forearm skin reactive hyperemia response is affected by the occlusion duration. Three minutes occlusion period is probably the optimum duration to use in studies of skin reactive hyperemia because it produced

the maximum postocclusive forearm skin reactive hyperemia compared to 1 and 2 minutes occlusion. Longer occlusion duration may not suitable as it may compromise subject's comfort (Tee and Rasool *et al.*, 2004).

The objective of this study is to establish the reproducibility of laser Doppler fluximetry for the assessment of postocclusive forearm skin reactive hyperemia in humans. Specifically, this study is to investigate the intra and interday variability using this method.

Methods

Subjects

Eighteen young, healthy, non-smoking, male volunteers participated in this study. All subjects underwent a screening program consisting of a full medical examination and hematologic and biochemical profiles. Those having bleeding disorder, Raynaud's phenomenon, personal history of hypertension, diabetes, hypercholesterolemia or other peripheral vascular diseases were excluded from the study. None took vitamin supplements or vasoactive drugs. Subjects must have a body mass index of less than 30kg/m^2 and arm circumference of less than 34cm. All subjects had normal full blood count and serum cholesterol level. Following explanation of the study protocol, its benefits and risks, subjects signed a written informed consent. The study was conducted according to the principles outlined in the Declaration of Helsinki of the World Medical Association, and the protocol was approved by the Universiti Sains Malaysia Research and Ethic Committee.

Principle and Technique of laser Doppler fluximetry

DRT4 laser Doppler fluximetry (Moor Instruments, Axminster, U.K.) used in this study is a non-invasive device that permits real-time continuous measurement of microvascular perfusion. In the present study, this instrument was used together with DP1T-V2 skin laser probe (Moor Instruments, Axminster, U.K.), which was stably held by PH1-V2 probe holder (Moor Instruments, Axminster, U.K.). Laser Doppler fluximetry generates a low intensity beam of monochromatic coherent 780nm light through an inbuilt infrared semi-conductor laser diode. This light was delivered to the site of measurement by a flexible fiberoptic probe. The laser light usually penetrates to a depth of 1.5mm [12].

Laser Doppler fluximetry uses Doppler shift principle. Photons of laser light scattered in moving blood cells produce a Doppler shift on the reflected light. This reflected light is detected by a photodetector and the signal is processed to determine the amount of the frequency shift. The recorded Doppler-shift signal corresponds to an average blood cell velocity obtained under an averaged angle. It also depends on the number of red blood cells in the sample volume. The resulting signal is therefore a product of the number of blood cells moving in the sample volume and the mean velocity of the moving blood cells. This outcome is generally termed as "flux".

Flux = Blood cell volume fraction x velocity

As the laser Doppler fluximetry measures blood flux, not blood flow, we hypothesized that the changes in blood flux measured reflected changes in blood flow. The measurement of blood flux is expressed in Arbitrary Unit (AU).

Inbuilt microprocessor in the DRT4 laser Doppler fluximetry allows measurement of perfusion over any specified time period. Besides measuring skin blood flux, the DP1T-V2 probe used also monitors skin temperature of the measurement site.

Protocol for skin blood flux measurement

Prior to each study session, subjects fasted overnight for at least 10 hours and avoided from consuming high salt food for 12 hours. They were also told to refrain from any heavy activities or vigorous exercises for 12 hours and withheld alcohol and caffeine-containing beverages for at least 48 hours. Subjects had familiarization visits with the instruments and study environment before the actual study.

All subjects were examined between 1200 and 1600 in a quiet and temperature-controlled room (room temperature was 24^0C with 70% relative humidity). Before laser-Doppler measurements were made, they were instructed to

rest supine for at least 15 minutes. During this acclimatization period, arms and hands of subject were kept stationary with the right arm uncovered. The right arm was supported by a hand supporter to reduce involuntary hand movements from influencing measurement.

Two laser probes (DP1T-V2 Skin Probe and P8a Low Profile Probe, Moor Instruments, Axminster, U.K.) were fixed separately on the volar surface of the right antebrachium, distal to a sphygmomanometer cuff (Accoson, England) placed around the right upper arm at 1-2cm above antecubital crease (Figure 1). Locations of the laser probes were marked, measured and recorded carefully, so as to be used identically on each study sessions. Stable baseline skin blood flux was recorded for 1 minute. Suprasystolic pressure (200mmHg) was insufflated into the pressure cuff in order to ensure full occlusion of the blood flow and maintained for 3 minutes. The occlusion was later released rapidly and reactive hyperemia was recorded for a duration of 2 minutes. The procedure was repeated after 15 minutes rest. No conversation was allowed throughout the measurement.

To address intraday and interday reproducibility, postocclusive forearm skin reactive hyperemia was performed twice within each study days for 2 study days. Intraday assessments were separated by 60-90 minutes, while the interday assessments were done 24-72 hours apart. All measurements were performed by the same operator throughout the whole investigation. As the variability of laser Doppler perfusion during resting is known to be substantial, the postocclusive forearm skin reactive hyperemia response was calculated (Absolute change in skin blood flux = peak skin blood flux minus the minimum baseline skin blood flux, in AU) in order to assess the reserve capacity of skin blood supply.

Statistical analysis

All results were presented as mean+/-SEM (standard error of mean). Statistical analyses were performed using SPSS Software version 11.0 (SPSS Inc., Chicago, IL, U.S.A). A combination of the Shapiro-Wilks test and normal distribution plots showed that data were normally distributed.

For each subject, within subject coefficient of variation was calculated as the standard deviation of all measurements, and divided by the mean for that subject. All individual coefficients of variations were pooled and reported as average for both intraday and interday measurements. Intraclass correlation coefficient with 95% confidence interval was also determined for both intraday and interday. Additionally, paired t-test was performed to test the differences between readings for both intraday and interday. Statistical significance was adopted as $p<0.05$.