REVIEW

A Review of Methods Currently Used for Assessment of 
In vivo
Endothelial Function

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An intact vascular endothelium is critical to the maintenance of normal arterial tone and coagulation status. Endothelial injury leading to dysfunction is thought to be a precursor to most if not all vascular disease, and has been implicated as a critical event in atherosclerosis. At present there are several methods available for detection of in vivo endothelial function, and the aim of this study was to critically review these methods. Five distinct methods were identified and studied in detail. These methods are diverse and each assesses a different vascular bed. Importantly there is no uniformity among investigators over choice of method and protocol, making it difficult to compare in vivo endothelial dysfunction between groups. These issues need to be addressed in large scale comparative analyses so that investigators can agree a common approach to endothelial function assessment.

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Introduction

An intact vascular endothelium is essential in maintaining short term control of arterial tone and coagulation status, and longer term control of smooth muscle cell proliferation and extra-cellular matrix production. Injury to the vascular endothelium is likely to be a preliminary event in most if not all vascular disease. Endothelial dysfunction has been implicated in a wide range of diseases from diabetes mellitus and essential hypertension, to vasospastic conditions such as systemic sclerosis and primary Raynaud’s phenomenon. Furthermore, it is postulated that endothelial dysfunction is a precursor to frank atherosclerosis;1–4 indeed it has been identified in vivo in healthy individuals exposed to cardiovascular risk factors such as cigarette smoking, obesity, increasing age and male sex. Cardiovascular disease is currently a leading cause of morbidity and mortality in the Western world,5 a fact which has provided a strong impetus for the development of methods that facilitate in vivo assessment of endothelial function. For many, the long term goal is none other than the establishment of a diagnostic tool that can detect disease early and monitor therapeutic responses.

There are at least five distinct methodologies that are currently being used for in vivo endothelial function assessment (Table 1). Some methods, such as venous occlusion plethysmography (VOP), are well established and have been in use for many years, while others are still at the developmental stage. Importantly, there appears to be very little uniformity among investigators regarding method selection and protocol. The aim of this study, therefore, is to critically review the methods currently being used for peripheral endothelial function assessment.

Venous Occlusion Plethysmography

Venous occlusion plethysmography (VOP) has been used to study forearm blood flow for many years. In
fact, it was first described in 1909 by Hewlett and van Zwaluwenburg, and aside from the incorporation of computer technology, the method has remained essentially unchanged since then. The underlying principle involves the arrest of venous outflow from the forearm such that it begins to swell. The rate and degree of swelling reflects forearm vascular resistance, which is a function of normal vascular endothelial function.

The method is widely used, with most practitioners using the protocol established by Wilkinson and Webb. When performing the procedure it is critical that baseline vasomotor tone remains constant, so factors which may affect vasoreactivity are carefully avoided. Thus, VOP is performed in a quiet, temperature-controlled room, with the subject relaxing in a reclining position. Subjects are asked to abstain from fatty meals, alcohol, caffeine and tobacco in the preceding 6 h. Blood pressure cuffs are placed around the arm and around the wrist, and the arm is held above the level of the heart. The arm cuff is inflated just enough to occlude venous outflow while preserving arterial inflow (around 40 mmHg), and the hand is excluded from the circulation by inflating the wrist cuff to supra-systolic pressures (200 mmHg). As the isolated forearm begins to swell, forearm volume, measured by a voltage dependent strain-gauge, increases in direct proportion to forearm blood flow. The hand is excluded because it’s blood flow is highly temperature sensitive, and it contains a high proportion of arterio-venous shunts. A degree of hand ischaemia is inevitable, and limits the testing period to usually no more than 10 min. Both arms are usually studied at the same time, the contra lateral arm providing a contemporaneous control. The increase in forearm volume is taken to represent blood flow in the resistance vessels of the forearm (muscle, soft tissues and skin).

VOP provides a convenient platform for testing forearm vascular resistance. In turn, vascular resistance can be readily manipulated by administering vasoactive drugs directly into the brachial artery using a small (27 G) cannula. Changes in vascular resistance reflect endothelial function. Investigating pharmacological effects in a closed circuit in this fashion has the advantage of avoiding systemic infusions of potentially dangerous drugs, and although there is a theoretical risk of critical forearm ischaemia when investigating vasoconstrictors, this is seldom seen in practice. Complications arising from repeated brachial arterial cannulation are rare, nevertheless the procedure should be regarded as moderately invasive.

Over the years, VOP has proved itself to be a robust and reliable tool for the investigation of vascular function. Despite an image of being cumbersome and somewhat dated, the procedure is well tolerated and continues to be popular. Using intra-arterial infusions of acetylcholine and sodium nitroprusside, VOP has been used to associate endothelial dysfunction with a range of cardiovascular risk factors such as smoking, hypertension, diabetes and aging. Most authors have found good reproducibility, and some even regard the technique as the ‘gold-standard’ for the assessment of vascular function.

Brachial Artery Flow-mediated Dilatation

Blood vessels have the capacity to adjust blood flow in response to luminal physical and chemical stimuli. This ability to self-regulate vasomotor tone allows the vessel to respond to changes in the local environment. An increase in blood flow will result in an increase in the ‘shear stress’ to which the local vascular endothelium is subjected and the vessel responds by dilating, a phenomenon called flow-mediated vasodilatation (FMD). Using a high resolution ultrasound scanner, it is possible to monitor and record
changes in vessel diameter resulting from FMD, and thus non-invasively determine endothelial function.

FMD is measured in conduit arteries, usually of a diameter between 2.5 and 5 mm, and the brachial artery is a convenient choice. The size of the vessel is an important consideration; if too small then it will be technically difficult to obtain accurate and reproducible images, whereas if too big then vasodilatation may be difficult to perceive, even when endothelial function is normal. A high-resolution ultrasound scanner with a high-frequency vascular transducer and an internal ECG monitor is required. The scanner must be equipped with appropriate vascular software for 2-D imaging, colour and spectral Doppler. Subjects are examined supine, with the arm held in a comfortable position, and care must be taken to avoid vasoreactive factors. The brachial artery is examined in the longitudinal plane above the antecubital fossa (Fig. 1). It is essential that a steady image of the artery is maintained throughout the study, and the relative position of anatomic landmarks such as veins and fascial planes are noted.

A blood pressure cuff, placed either above or below the transducer position, is used to create the flow stimulus in the brachial artery. When placed above, the stimulus is greater, but accurate visualisation is more difficult. After a baseline diameter measurement, the cuff is inflated to a suprasystolic pressure for 5 min. Cuff deflation induces a brief high flow state (reactive hyperaemia) that subjects the endothelium to shear stress causing it to dilate. This is said to be to be endothelium-dependent vasodilatation. FMD is calculated as the percentage increase in diameter from baseline to maximum post-cuff deflation diameter. After a 10 min rest period, the artery returns to the baseline diameter, at which point sublingual glyceryl trinitrate (GTN) is given. The maximum diameter after GTN reflects smooth muscle integrity (endothelium-independent vasodilatation) and is theoretically the maximum vasodilator response.

While some investigators have found the technique to be both accurate and reproducible, others have not been so impressed. There is no doubt that FMD is vulnerable to criticisms of reproducibility, and intra/inter-observer variability. An International Brachial Artery Reactivity Task Force has been appointed, and steps to investigate and minimise these problems are underway. Ultrasonographic assessment of the brachial artery is uniquely challenging and the examination must be performed by well trained, skilled individuals. There is a significant learning curve, and once sufficiently trained, sonographers must continue to perform the technique on a regular basis to maintain their skills. Ergonomic factors are also important, with both examiner and subject assuming comfortable positions, such that the transducer remains steady at the same anatomical position throughout. This may seem straightforward, but is actually quite a technical challenge, and the use of a stereo tactic probe holding device has been advocated (and should probably be considered mandatory). Even when excellent images of the brachial artery are obtained, off-line diameter measurement using ultrasound callipers is not reliable, and should be regarded as out of date. Some investigators now use radio-frequency processing software, as this facilitates accurate on-line tracking of the anterior and posterior arterial walls. To account for diameter changes associated with the cardiac cycle, the signal must be ECG gated. Systems of this type can give a theoretical accuracy of the order of 5 μm, although this is highly dependent on the quality of the image.

The theory and methodology behind FMD measurement is now well established, and most vascular laboratories are already equipped with the necessary hardware. One can anticipate improvements in the technique in parallel with advances in imaging technology and computer software; for example, phase-contrast magnetic resonance angiography has been advocated as an alternative to high-resolution ultrasound. Although this may prove superior in detecting minute vessel diameter changes, at present ultrasound is preferred as it is more dynamic (and cheaper).

Iontophoresis in Conjunction with Laser Doppler Imaging

Laser doppler iontophoresis (LDI) is a convenient and
simple technique for transdermal administration of a minute quantity of a drug, using small electric currents. The underlying principle is that the molecules of a drug in solution are either positively or negatively charged, and will migrate across the skin under the influence of an applied monopolar current. The rate and quantity of drug delivered is dependent on the magnitude of the applied current and it’s duration:

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\text{current (Amps)} \times \text{time (seconds)} = \text{charge (Coulombs)}.
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This method of drug delivery is not new, and has been in use for over 20 years. However, it has only recently become apparent that, when used in conjuction with laser Doppler flowmetry, it is a useful tool for monitoring microvascular endothelial function. The procedure involves time-controlled delivery of a vasoactive drug onto a patch of skin on the subjects forearm. The resultant alterations in skin blood flow are then detected using laser Doppler flowmetry, or more recently using a laser Doppler imager. Laser Doppler imaging allows blood flow measurement over the entire distribution of the administered drug, whereas laser Doppler flowmetry restricts blood flow measurements to single points in the distribution. The alterations in blood flow reflect endothelial function at a microvascular level. A typical equipment set up is shown in diagrammatic form in Fig. 2.

Acetycholine and sodium nitroprusside are used to generate endothelium-dependent and endothelium-independent vasodilatation, respectively. The procedure is carried out using very small currents (less than 100 μA), and is painless. The forearm microvascular bed is usually the site of choice, and iontophoretic electrodes are attached to the volar aspect. The quantity of drug delivered is too small to have any systemic effects, although mild allergic reactions and skin irritation have been reported. The procedure is performed in similar conditions to those described for VOP and FMD, taking care to avoid factors that may induce vasoreactivity.

LDI is an attractive technique, but there are several important problems that must be overcome. The variability of skin conductivity in different populations must be considered when designing studies and interpreting results. While Ohm’s Law has been used to correct for these differences, one could argue that applying such physical laws to organic tissue adds a spurious air of accuracy. Although the procedure is short (typically 15 min), subjects are required to remain entirely still during that time, with the forearm held in supination; this may result in movement artefacts owing to subject fatigue.

A more significant problem is the tendency of the applied current itself to cause vasodilatation, even in the absence of an administered drug. The precise aetiology of current-induced vasodilatation is complex and has yet to be fully defined. It has been well documented that this phenomenon is more pronounced with cathodal (acetylcholine) rather than anodal (sodium nitroprusside) drug delivery. Some investigators have postulated that primary afferent nerves, specifically C-nociceptive fibres, may have an important role. Neurovascular responses are thought to be directly stimulated by the applied current, resulting in the release of vasodilatory neuropeptides, such as calcitonin gene-related peptide and substance P. The most effective way of limiting current-induced vasodilatation seems to be the use of smaller currents over longer time-periods. The total charge (and drug dose) delivered remains the same, but stimulation of the neurovascular response is less pronounced.

LDI is becoming increasingly popular, and with good reason. It is non-invasive, provides a direct assessment of microvascular endothelial function, and the technique is resistant to accusations of observer dependency. Reproducibility should not be a problem if precautions, such as strict standardization of the recording site, are taken.

**Pulse-wave Analysis**

LDI and FMD detect endothelial dysfunction in local vascular beds, either skin microvasculature or the brachial arterial tree. Pulse-wave analysis is a relatively new technique that provides a non-invasive method of assessing global endothelial function. Arterial stiffness is partly dependent on vasomotor tone, which in turn relies on an intact endothelium. As the arterial pulse waveform travels from the central circulation to the periphery, it’s shape provides a measure of systemic arterial stiffness, thus changes in the shape of the waveform will partly reflect endothelial function. The waveform is readily observed at the radial artery by applanation tonometry. The arterial pulse waveform is reflected from the periphery back to the central circulation, and this may confound the observed radial waveform. To account for this, endothelial function is assessed by changes in the augmentation index (AIx), which is a function of the relationship between the reflected arterial wave and the primary aortic wave. In essence, AIx provides a measure of systemic arterial
stiffness, and several studies have used it as a surrogate marker for endothelial function.\textsuperscript{35,36,40,42} Endothelium-dependent vasodilatation is assessed following inhalation of the β2-adrenergic receptor agonist salbutamol, which has been shown to cause the release of endothelium-derived vasodilator nitric oxide.\textsuperscript{43} Endothelium-independent vasodilatation is assessed following intravenous administration of GTN.

The technology required for PWA is easily available and relatively cheap, and it is relatively immune to criticisms of observer dependency. But the procedure is still at an early developmental stage and is not widely used. Also, more work needs to be done to clarify whether AIx is a consistently reliable parameter. Those with high cardiovascular risk or established atherosclerotic disease may exhibit different wave reflection characteristics from the groups studied thus far. Similarly, baseline cardiac parameters may preclude certain patients from study. A high resting heart rate, for example, may have an independent association with arterial stiffness.\textsuperscript{44} In fact, the precise association between arterial stiffness and endothelial function has yet to be elucidated.

**Retinal Arterial Abnormalities**

Abnormalities of architecture in an arterial network can reflect generalised circulatory efficiency. The retina
has long provided a convenient arterial bed in which it is possible to make non-invasive, in vivo assessments of arterial architecture. The presence of abnormalities, such as focal and generalised narrowing, arteriovenous nicking, altered arteriole to venule ratio, and suboptimal arterial diameter at bifurcations, can be suggestive of a more generalised circulatory disorder.\textsuperscript{45–47} It has been reported, most notably by the atherosclerosis risk in communities (ARIC) study, that abnormalities in retinal arterioles are related to the presence of carotid plaque, hypertension, and serum markers of inflammation and endothelial function such as von Willebrand Factor.\textsuperscript{46,47} Although it has been reported that inhibition of endothelium-derived nitric oxide synthesis by the nitric oxide synthase antagonist NG-monomethyl-l-arginine (l-NMMA) induces changes in junction exponents in human retinal arteries,\textsuperscript{48} a precise correlation between peripheral endothelial dysfunction and retinal arterial abnormalities has yet to be made. A correlation has, however, been made between the presence of peripheral vascular disease and retinal arteriolar abnormalities.\textsuperscript{48}

The retinal arteriolar network can be assessed using non-invasive scans, which are then assessed by trained individuals, assisted by computer software. The concept of a simple retinal scan providing important information about cardiovascular risk in individuals is highly attractive. There seems no doubt that established cardiovascular disease, probably with widespread haemodynamic impairment, could be reflected in retinal arterial architecture. However, as far as we are aware, no group has yet demonstrated that generalised endothelial dysfunction per se, can result in retinal arterial abnormalities.

**Clinical Impact**

Of the methods described in this review, by far the most widely used are VOP, FMD and LDI. Each one of these methods assesses endothelial function in a different vascular bed, and they vary in their degree of invasiveness (VOP, requiring arterial cannulation should be regarded as more invasive than either FMD or LDI). The choice of which method to use is largely governed by local knowledge and equipment availability, and there is as yet no data to suggest that any particular method should be used in any particular study. An important question is—does endothelial dysfunction in one vascular bed correlate with endothelial function in other vascular beds in the same subject group?

Several investigators have attempted to answer this question, but their reports are conflicting. In a somewhat limited study of six healthy subjects compared to 10 hypertensive/obese individuals, Irace et al.\textsuperscript{49} reported a high correlation of endothelial function ($p < 0.001$) between FMD and VOP. However, Lind et al.\textsuperscript{50} evaluated both these methods in 24 healthy subjects, and did not find any correlation. Raitakari et al.\textsuperscript{21}, in their study of post-prandial endothelial function, noticed a significant change with VOP, but not with FMD ($p < 0.001$; $n = 12$). Similar studies comparing FMD and LDI also fail to demonstrate a significant correlation in endothelial function in these vascular beds.\textsuperscript{51,52} PWA has been widely used to study arterial stiffness, but it’s use for endothelial function testing is still limited. However, comparisons between PWA and VOP appear to be quite favourable, as confirmed by Lind et al.,\textsuperscript{50} and also by Wilkinson et al.\textsuperscript{36}

Perhaps the most compelling question is—can any of these methods be used in individuals as a screening tool for cardiovascular disease? Alas, at present the answer appears to be no. The very nature of endothelial function testing makes it incompatible with good reproducibility in individuals, because it’s extremely difficult to reliably control all potentially vasoreactive influences at all times. Furthermore, some of the methods described (such as FMD) are inherently prone to observer error. However, these methods are useful when studying the effect of a single risk factor or condition on a group of individuals. Thus, more work is required to inform future investigators on the appropriate choice of method/-protocol, and there needs to be a better degree of uniformity so that findings are easily compared.

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