Video Article

Non-invasive Assessment of Microvascular and Endothelial Function

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Abstract

The authors have utilized capillaroscopy and forearm blood flow techniques to investigate the role of microvascular dysfunction in pathogenesis of cardiovascular disease. Capillaroscopy is a non-invasive, relatively inexpensive methodology for directly visualizing the microcirculation. Percent capillary recruitment is assessed by dividing the increase in capillary density induced by postocclusive reactive hyperemia (postocclusive reactive hyperemia capillary density minus baseline capillary density), by the maximal capillary density (observed during passive venous occlusion). Percent perfused capillaries represents the proportion of all capillaries present that are perfused (functionally active), and is calculated by dividing postocclusive reactive hyperemia capillary density by the maximal capillary density. Both percent capillary recruitment and percent perfused capillaries reflect the number of functional capillaries. The forearm blood flow (FBF) technique provides accepted non-invasive measures of endothelial function: The ratio FBF_{max}/FBF_{base} is computed as an estimate of vasodilation, by dividing the mean of the four FBF_{max} values by the mean of the four FBF_{base} values. Forearm vascular resistance at maximal vasodilation (FVR_{max}) is calculated as the mean arterial pressure (MAP) divided by FBF_{max} . Both the capillaroscopy and forearm techniques are readily acceptable to patients and can be learned quickly.

The microvascular and endothelial function measures obtained using the methodologies described in this paper may have future utility in clinical patient cardiovascular risk-reduction strategies. As we have published reports demonstrating that microvascular and endothelial dysfunction are found in initial stages of hypertension including prehypertension, microvascular and endothelial function measures may eventually aid in early identification, risk-stratification and prevention of end-stage vascular pathology, with its potentially fatal consequences.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50008/>

Protocol

Case Presentation (required, if applicable): N.A.; this is still an experimental research procedure, not yet used clinically.

Diagnosis, Assessment, and Plan (required, if applicable): N.A.; this is still an experimental research procedure, not yet used clinically.

Procedure (required): This part should include a step-by-step description of relevant procedures, meeting the guidelines below.

1. Capillary Microscopy (Figure 1)

1. Our capillaroscopy technique was adapted from Serne and his colleagues¹. An exclusion criterion for this procedure is collagen vascular disease, since collagen vascular disease produces known capillary changes².
2. Following a minimum 10-hr overnight fast and 20 min of seated rest, microvascular measurements are conducted for one half-hour between 7 and 11 am, in a quiet, temperature controlled room (maintained between 21.5-22.5 °C), with the subject in the seated position and the left hand at heart level.
3. Nailfold capillaries in the dorsal skin of the third finger are visualized using a stereomicroscope (Olympus; Center Valley, PA), linked to a 4 megapixel SPOT Insight monochrome digital camera (Model number IN-1400: Diagnostic Instruments; Sterling Heights, MI), and a laptop computer (Dell Latitude D600: Dell; Austin, TX). To limit movement, the left hand and forearm are loosely covered with a folded blanket, and rested on another folded blanket positioned at the base of the microscope.
4. Nailbed illumination is achieved with a 250-W halogen fiber optic lamp (KL 2500LCD:Schott-Fostec; Elmsford, NY); additional illumination from a supplemental 150W fiber optic halogen light source (B&B Microscopes, Ltd., Warrendale, PA) is used in darkly pigmented individuals.

5. To visualize the capillaries, the 3.2x objective (Olympus 3.2/0.07) is used with a total system magnification of 38.4x.
6. Using SPOT imaging software provided with the camera, light/dark contrast in the capillary photographs is enhanced using the same standard SPOT software function (stretching of bright and dark levels) to maximize visibility of the capillaries in all subjects.
7. To quantify capillary density, digital photomicrographs are taken every 3-5 sec during each of three stages, at resting baseline, during postocclusive reactive hyperemia, and during venous occlusion. (a) At resting baseline, photomicrographs are taken over a three-minute period to detect capillaries perfused at rest. (b) During postocclusive reactive hyperemia, photomicrographs are taken to quantify functionally perfused capillaries (baseline plus reserve capillaries), as follows. First, an occlusion cuff on the left upper arm is inflated to 40 mm Hg above systolic pressure for 10 min. Photomicrographs are then taken during the first minute immediately following release of arterial occlusion, visualizing all functionally perfused capillaries. Lower capillary density following reactive hyperemia indicates impaired functional capillary recruitment, and therefore functional rarefaction. (c) During venous occlusion photomicrographs are taken to quantify maximal capillary density, which includes both perfused (with active red blood cell (RBC) motion) and nonperfused (filled with stagnant, non-moving RBCs) capillaries³ as follows. Following ten minutes of rest after the postocclusive reactive hyperemia procedure, the arm cuff is inflated to 60 mm Hg for 60 sec, passively forcing blood into all patent capillaries present and photomicrographs were taken during this time. Since maximal capillary density includes all capillaries structurally present, a reduction in maximal capillary density indicates structural rarefaction.
8. Capillary density is defined as the number of capillaries per square millimeter of nailfold skin, and is computed as the mean of four measurements obtained from the four most clearly focused images, least distorted by movement. In our studies, typical values for capillary counts (capillaries/mm²) have been 55-80 for baseline, 65-90 for post-ischemic, and 90-105 for venous occlusion. Values for percent capillary recruitment are typically between 5% and 25% (mean ~10-15%) and for percent perfused capillaries between 70% and 95% (mean ~80-90%), with values being lower among hypertensives than normotensives. The reproducibility of the counting procedure has been verified with three observers who performed independent manual assessments of photographs of 10 different subjects (**Figure 2**). The observers were blinded to the identity and blood pressure of these subjects. Following training, subsequent counts performed independently showed a high level of agreement. Average inter-rater and intra-rater discrepancies were of the order of 2-3 capillaries/mm², and intraclass correlation coefficients were all greater than 0.90. Short-term variation of the capillaroscopy measures were of the same order of magnitude as inter-rater and intra-rater discrepancies (approximately 2 capillaries/mm²), but longer-term variation observed over 2-3 years was an order of magnitude larger (approximately 15 capillaries/mm²), indicating that longitudinal changes can be readily distinguished from rater variation. Reliability of the two capillary function measures was also high (intraclass correlation coefficient = 0.84 for percent capillary recruitment and 0.82 for percent perfused capillaries).
9. The investigators now utilize a computer-based method for quantifying capillary density using Image-Pro Plus imaging software (Version 6.2, Media Cybernetics, Inc., Bethesda, MD: **Figure 3**). Pearson correlations between baseline, post-ischemic, and venous congestion counts done with the software and corresponding manual counts in 10 subjects were 0.78, 0.78, and 0.71 respectively (all $p < 0.05$), indicating reasonable agreement between the two methods. Reliability of the computer-based counts is slightly lower than that of manual counts but still high (intraclass correlation coefficient = 0.91 for baseline, 0.86 for post-ischemic, and 0.84 for venous occlusion). We have unpublished data also demonstrating the association of automated counts with multiple cardiovascular risk factors including hypertension, which we are currently preparing for publication.
10. **Table 1** summarizes the capillary density measurements and calculations. Percent capillary recruitment is assessed by dividing the increase in capillary density induced by postocclusive reactive hyperemia (postocclusive reactive hyperemia capillary density minus baseline capillary density), by the maximal capillary density (observed during passive venous occlusion). Percent perfused capillaries represents the proportion of all capillaries present that are perfused (functionally active), and is calculated by dividing postocclusive reactive hyperemia capillary density by the maximal capillary density. Both percent capillary recruitment and percent perfused capillaries reflect the number of functional capillaries. Lower values for these measures indicate functional capillary rarefaction.

2. Endothelial Function Assessment

1. Endothelial function is assessed before and after postocclusive reactive hyperemia, using non-invasive plethysmography measurements of forearm blood flow, according to the method of Sivertsson,⁴ which utilizes the endothelium-dependent stimulus of reactive hyperemia to induce vasodilation.
2. With the subject in the seated position following 10 min of supine rest, a mercury-in rubber strain gauge stretched to 10% beyond its resting length is looped around the subject's forearm 5 cm below the antecubital fossa.
3. The strain-gauge is connected to a plethysmograph (EC-4: DE Hokanson, Inc; Bellevue, WA), which in turn is connected to a Doppler recorder (CW-1; DE Hokanson, Inc; Bellevue, WA).
4. An upper arm occlusion cuff is applied, and the arm is suspended comfortably at heart level using a sling bandage connected to an adjustable intravenous pole. Systolic and diastolic blood pressures and heart rate are obtained with a Dinamap ProCare 100 automatic BP cuff (GE Healthcare, Piscataway, NJ) placed on the opposite arm.
5. A pediatric cuff around the wrist is inflated to 200 mm Hg to occlude flow to the hand. The upper arm cuff is inflated to 50 mm Hg, deflated for 1.5 sec, and then re-inflated rapidly prior to each forearm blood flow measurement, obtained through expansion of the strain gauge placed around the forearm.
6. Forearm blood flow (FBF) is measured at rested baseline (FBF_{base}) and again at postocclusive hyperemia-induced maximal vasodilation (FBF_{max}). For baseline blood flow measurements, four consecutive FBF curves are obtained within 30 sec (FBF_{base}).
7. The occlusion cuff is then inflated to 40 mm Hg above systolic pressure for 10 min. Following release of arterial occlusion (postocclusive reactive hyperemia), four consecutive FBF curves are obtained within the first 30 sec of flow (FBF_{max}).
8. The ratio FBF_{max}/FBF_{base} is computed as an estimate of vasodilation, by dividing the mean of the four FBF_{max} values by the mean of the four FBF_{base} values.⁵ Forearm vascular resistance at maximal vasodilation (FVR_{max}) is calculated as the mean arterial pressure (MAP) divided by FBF_{max}. FBF_{max} during reactive hyperemia is directly related to FBF after maximum infusion of intra-arterial acetylcholine, an endothelial-

dependent vasodilator.⁶ Accordingly, FBF_{max} and the ratio FBF_{max}/FBF_{base} are accepted non-invasive measures of endothelial function.⁶⁻⁸ In addition, both FBF_{max} and FVR_{max} reflect resistance artery structural changes (increased wall/lumen ratio).⁹

Representative Results

Differences in the appearance of the microvasculature between normotensive and hypertensive individuals are readily apparent by comparing **Figures 4 and 5**. **Figure 4** shows the typical network of straight capillaries in well organized rows in a normotensive individual. In contrast, **Figure 5** demonstrates a more disarranged pattern of shrunken, coiled capillaries.

The authors have an ongoing interest in the role of microvascular dysfunction in pathogenesis of cardiovascular disease. In their first NIH/NHLBI grant, the authors successfully assembled a cohort of 200 individuals, including normotensive, prehypertensive, and hypertensive subjects, and performed a series of investigations on microvascular dysfunction (capillary rarefaction and endothelial function measures). Individuals in this cohort ranged in age from 18-55, were 60% female, 46% African American, and 61% prehypertensive (n=122). We have published reports from this cohort demonstrating that microvascular dysfunction is found in initial stages of hypertension (prehypertension and Stage 1 hypertension: details previously published.^{10, 11} While cross-sectional, our findings indicate that the hypertensive vascular pathologic process is already underway at modest levels of blood pressure elevation, suggesting that microvascular dysfunction may predate development of chronic sustained hypertension.

Capillary density = number of capillaries per square millimeter (mm^2) of finger nailfold skin

- A. **Resting baseline:** continuously perfused capillaries¹
- B. **Postocclusive reactive hyperemia:** continuously perfused + intermittently perfused (functional reserve) capillaries; measure of capillary function¹
- C. **Venous occlusion (maximal capillary density):** maximal visualization of all capillaries present, including both perfused (with active red blood cell (RBC) motion) and nonperfused (filled with stagnant, non-moving RBCs) capillaries; measure of capillary structure³

Percent capillary recruitment = $(B-A) \div C \times 100$

[Postocclusive reactive hyperemia capillary density - resting baseline capillary density]

÷

Maximal capillary density (during passive venous congestion)

Measure of capillary function

Percent perfused capillaries = $(B+C) \times 100$

Postocclusive reactive hyperemia capillary density

÷

Maximal capillary density (during passive venous congestion)

Measure of capillary function

Table 1. Measures of capillary structure and function.

Figure 1. Capillaroscopy. diagnostic examination of capillaries, especially of the nail beds, with a microscope.

Figure 2. Capillaroscopy images. Counting by hand. Boxed area noted is drawn using a Staedler engineering ruler and denotes 1 square millimeter.

Figure 3. Computer-Assisted Capillaroscopy. Image-Pro software is used to enhance contrast (example on the right) in the original digital images (example on the left), which can then be counted using the software.

Figure 4. Capillaroscopy image: Normotensive individual.

Figure 5. Capillaroscopy image: Hypertensive individual.

Discussion

Capillaroscopy (capillary microscopy) is a non-standard measure of capillary structure. However, currently, there are no standard methods for direct assessment of capillary structure. Furthermore, capillary microscopy has been widely used for the direct evaluation of capillary density in a large and growing body of published work^{12, 13, 10, 11, 14-18}. Additionally, we have validated the capillary microscopy technique by correlating capillaroscopy findings with forearm blood flow, a well-established measure of vascular function,^{10, 11} and with UAE, a well-established measure of vessel injury (unpublished work, manuscript in preparation). While the original methodology utilizes continuous visualization using video photography, our simplified methodology utilizes still digital photographs taken repeatedly over minutes as detailed in the procedures section.

While it is theoretically true that peak capillary density could be underestimated or missed using still images to count capillaries without a running videotape to refer to, we utilize the still images taken in rapid succession for 1 minute during peak reactive hyperemia following release of the cuff, specifically counting the first four pictures thus obtained, which should provide the maximal post-ischemic capillary counts. Furthermore, it has been reported that by using a prolonged period of upper arm ischemia at suprasystolic pressure for several minutes, the hyperemia response may last for 3 to 4 minutes in healthy subjects at least, as opposed to only 10 seconds following 1 minute occlusion with a finger cuff.¹⁹

We made adaptations to the technique of Serne *et al.* for two reasons: 1) Primarily to make the technique more practical and accessible for use in both a clinical and clinical research setting, and 2) To more closely mirror the methodology we have used to validate the technique by comparing our results to those found in the forearm plethymography technique, which is an established method for assessment of vascular function. We used an arm cuff in place of digital cuffs, which are not readily available in the United States. Also as noted above, the use of upper arm versus digital ischemia may result in a more prolonged hyperemia response, lending itself more readily to capture of the peak hyperemia response for analysis. Placement of the ischemia cuff at the upper arm instead of the forearm results in greater hyperemia flow (resulting from higher brachial artery vasodilatation).⁶ In developing the technique, we researched the literature for the most appropriate period of arterial occlusion. We found periods ranging from 3 minutes¹⁹ to 5 minutes^{4, 6, 8} to 13 minutes,^{20, 21} with one of the studies using 13 minutes reporting good reproducibility of the values thus obtained. Yet another study showed increasing blood flow with increasing occlusion time.²² Accordingly, we chose 10 minutes as an intermediate value.

We have introduced the new parameter, **percent capillary recruitment**, which expresses capillary recruitment as a percentage of the maximal number visualized during venous occlusion, in an attempt to normalize the number of recruited vessels by dividing by the total number of capillaries present, enabling direct comparison of the total number of actively perfused (functional) capillaries between individuals. We have also reported the more widely used parameters (capillary recruitment and venous occlusion) in our publications to date.

Our technique utilizes a lower magnification (38.4 x) compared to that of others reported in previous literature. The capillary density reported by James/Shore *et al.*²³ (98-117 per mm²) and Antonios¹², who also uses high magnification 196x (57- 93 per 0.68 mm²) are indeed higher. However, Debbabi/Levy²⁴ obtained capillary densities (60-79) almost identical to ours (55-74), using 200x magnification. Our counts are actually higher than those reported by Serne *et al.*²⁵, who used 100x magnification (48-57). This may be at least partially due to the fact that the absolute number of vessels per field is reduced at higher magnification, making it more difficult to identify a reduced number of vessels in disease.

Additionally, our use of 38.4x magnification produces digital images from which we can obtain several adjacent clear fields⁴ without need of further processing (reduction or magnification) of the images.

In our investigations, we have elected to study capillaries in the peripheral circulation, which are readily accessible in the fingertips using a simple stereomicroscope, and can easily be studied without the use of intravenous dye injection.²⁶ It has been stated that the capillaroscopy technique is difficult to perform in highly pigmented subjects^{24, 27}, with no data on capillary rarefaction in black subjects reported prior to our findings. Using the dual light sources and photo-enhancing software described in the methods, we are able to visualize and quantify capillaries in all enrolled subjects, including darkly pigmented blacks.

In summary, capillaroscopy is a non-invasive, relatively inexpensive methodology for directly visualizing the microcirculation. Both the capillaroscopy and forearm techniques are readily acceptable to patients and can be learned quickly. The microvascular and endothelial function measures obtained using the methodologies described in this paper may have future utility in clinical patient cardiovascular risk-reduction strategies. As we have published reports demonstrating that microvascular and endothelial dysfunction are found in initial stages of hypertension including prehypertension, capillaroscopy and venous occlusion plethysmography may eventually aid in early identification, risk-stratification and prevention of end-stage vascular pathology, with its potentially fatal consequences.

Disclosures

The authors have no financial conflicts of interest related to the conduct or publication of this work to disclose.

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