Video Article

Assessment of Vascular Function in Patients With Chronic Kidney Disease

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Abstract

Patients with chronic kidney disease (CKD) have significantly increased risk of cardiovascular disease (CVD) compared to the general population, and this is only partially explained by traditional CVD risk factors. Vascular dysfunction is an important non-traditional risk factor, characterized by vascular endothelial dysfunction (most commonly assessed as impaired endothelium-dependent dilation [EDD]) and stiffening of the large elastic arteries. While various techniques exist to assess EDD and large elastic artery stiffness, the most commonly used are brachial artery flow-mediated dilation (FMD_{BA}) and aortic pulse-wave velocity (aPWV), respectively. Both of these noninvasive measures of vascular dysfunction are independent predictors of future cardiovascular events in patients with and without kidney disease. Patients with CKD demonstrate both impaired FMD_{BA}, and increased aPWV. While the exact mechanisms by which vascular dysfunction develops in CKD are incompletely understood, increased oxidative stress and a subsequent reduction in nitric oxide (NO) bioavailability are important contributors. Cellular changes in oxidative stress can be assessed by collecting vascular endothelial cells from the antecubital vein and measuring protein expression of markers of oxidative stress using immunofluorescence. We provide here a discussion of these methods to measure FMD_{BA}, aPWV, and vascular endothelial cell protein expression.

Video Link

The video component of this article can be found at http://www.jove.com/video/51478/

Introduction

Chronic kidney disease (CKD) is a major public health concern that has reached epidemic proportions, affecting ~11.5% of the population in the United States alone[1]. The risk of cardiovascular death or a cardiovascular event in patients with CKD is significantly increased compared with the general population[2-4]. Although patients with CKD exhibit a high prevalence of traditional cardiovascular risk factors, this only explains part of their increased incidence of cardiovascular disease (CVD)[5]. Vascular dysfunction is an important nontraditional cardiovascular risk factor gaining increased recognition in the field of nephrology[6-9].

While many changes likely contribute to the development of arterial dysfunction, among those of greatest concern are the development of vascular endothelial dysfunction, most commonly assessed as impaired endothelium-dependent dilation (EDD), and stiffening of the large elastic arteries[10]. Various techniques exist to assess EDD and large elastic artery stiffness, but the most commonly used are brachial artery flow-mediated dilation FMD_{BA} and aortic pulse-wave velocity (aPWV), respectively. Another commonly used technique to assess EDD is measuring forearm blood flow response to pharmacological agents such as acetylcholine using venous occlusion plethysmography[11,12]. However, this methodology requires catheterization of the brachial artery, which is more invasive than FMD_{BA} and may be contraindicated in patients with CKD. An alternate technique to assess arterial stiffness is to measure the local arterial compliance (the inverse of stiffness) of the carotid artery, although this is not as widely used or validated with clinical endpoints as aPWV[13] .

Patients with CKD demonstrate both impaired FMD_{BA}[14-16] and increased aortic pulse-wave velocity aPWV[13,17,18], even prior to needing dialysis. Importantly from a clinical perspective, both of these noninvasive measures of vascular dysfunction are independent predictors of future cardiovascular events and mortality both in patients with CKD[19-21], as well as in other populations[22-26]. These techniques can be applied to studying various populations at risk of CVD, including patients with CKD.

The exact mechanisms by which arterial dysfunction develops in CKD are incompletely understood; however, reduced nitric oxide (NO) bioavailability is a critical contributor[27-30] and a common mechanism of both impaired EDD and increased arterial stiffness[10,31]. In CKD, oxidative stress is increased and contributes to the reduction in NO bioavailability[32-34]. Oxidative stress is defined as excessive bioavailability of reactive oxygen species (ROS) relative to antioxidant defenses. Physiological stimuli, including inflammatory signaling, promote oxidant enzyme systems (*e.g.*, the oxidant enzyme NADPH oxidase) to produce ROS, including superoxide anion ($O_2\bullet^-$)[35]. Production of superoxide ultimately leads to reduces bioavailability of nitric oxide (NO).

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Impaired NO bioavailability may in turn contribute to the development of CKD, as endothelial dysfunction is an independent predictor of incident CKD[36]. This is consistent with animal data indicating that eNOS inhibition induces hypertension (systemic and glomerular), glomerular ischemia, glomerulosclerosis, and tubulo-interstitial injury[37]. Indeed, reduced NO bioavailability appears necessary for the development and progression of experimental kidney disease that mimics human disease, suggesting a key role for endothelial dysfunction in human CKD[38,39].

Markers of vascular oxidative stress can be assessed in vascular endothelial cells collected from human research subjects, using a technique originally developed by Colombo *et al.*[40] and modified Seals *et al*[41-43]. Using 2 sterile J-wires, cells are collected from the antecubital vein, recovered, fixed, and later positively identified as endothelial cells and analyzed for expression of proteins of interest using immunofluorescence.

We provide here a discussion of this methodology that can be used to a) measure FMD_{BA}; b) measure aPWV; c) measure vascular endothelial cell protein expression of markers of oxidative stress. The focus is on patients with CKD, not requiring chronic dialysis.

Protocol

This protocol follows the guidelines of the Colorado Multiple Institutional Review Board (COMIRB).

1. Preparation for Testing Session

1. Participants should follow these restrictions for most accurate measurements: 12 hr fast from food and caffeine, 12 hr restraint from exercise, 12 hr restraint from smoking, if applicable, ≥4 half-life restraint from medications if possible (may not be feasible in a population such as CKD patients), and pre-menopausal women should be tested in days 1-7 of the menstrual cycle to minimize hormonal influences.
2. Prepare 500 ml of the dissociation buffer by adding 2 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA), 0.05 g of heparin (180 USP units/mg), and 2.5 g of bovine serum albumin to 476.8 ml of phosphate buffered saline (PBS) at pH of 7.4. This can be stored at 4 °C for several months.
3. Turn on ultrasound, computer, and the Non-Invasive Hemodynamic Workstation (NIHem; arterial stiffness equipment). Connect cables outputting the ultrasound to the computer R-wave trigger box.

2. Collection and Processing of Vascular Endothelial Cells

1. A trained nurse or physician performs the collection (steps 2.2-2.5, 2.7) and a researcher collects and processes the wires (steps 2.6, 2.8-2.19)
2. Prep the antecubital site with a topical antiseptic, apply a tourniquet, locate vein, and cannulate with an 18 G catheter. Place a heplock adapter on the end of the IV.
3. Put on sterile gloves and put sterile fenestrated drapes over the site.
4. Place 2 J-wires on the drapes. Pull the arc of the "J" to uncoil the "J" shape from both wires.
5. Uncap the heplock and feed J-wire into vein approximately 8 cm. Push back and forth several times before removing the wire. Avoid gross blood on the wire.
6. Use wire cutters to snip the wires so they fit in a 50 ml conical tube containing ~30 ml of dissociation buffer
7. Repeat step 2.5 for second wire.
8. Repeat step 2.6 for second wire. Return tube to wet laboratory.
9. Clasp the wires with a pair of forceps and hold the wires inside of the tube, but above the solution. For 10 min, use a motorized pipetter to repeatedly collect the dissociation buffer from the 50 ml conical tube and release it so it runs down the length of the wires to rinse and vibrate the wires, then shake off excess fluid from wires into tube.
10. Centrifuge for 7 min at 400 x g and 4 °C.
11. Prepare the formaldehyde solution in foil covered tube by combining 100 ml formaldehyde solution + 900 ml PBS.
12. Slowly remove tube from centrifuge, turn on the vacuum pump, place a pipette tip on end of suction hose and leave ~400 ml in the tube, vacuuming off the rest **without disturbing the pellet.**
13. Cover with foil and pipette 1 ml formaldehyde solution into the tube to fix the sample. Do not resuspend. Incubate for 10 min at room temperature.
14. Prepare 8 slides by labeling with subject and study visit information and drawing an oval on each slide with a pap pen.
15. Add 15 ml PBS, resuspend, and centrifuge for 5 min at 400 x g and 4 °C.
16. Repeat step 2.15, add 12 ml PBS, resuspend, and centrifuge for 6 min at 400 x g and 4 °C.
17. Slowly remove tube from centrifuge, turn on the vacuum pump, place a pipette tip on end of suction hose and leave ~2 ml in the tube, vacuuming off the rest **without disturbing the pellet.**
18. Resuspend and pipette evenly across the 8 slides in the oval areas.
19. Place in incubator at 37 °C for 5 hr and then store at -80 °C until ready for analysis (samples will be fine for many years).

3. Assessment of FMD_{BA} and aPWV

1. Have research subject change into disposable shorts and have him/her lie supine in a quiet, dim, climate controlled room.
2. Place the appropriate number of ECG for the specific ultrasound and arterial stiffness device (this procedure uses the Non-Invasive Hemodynamic Workstation [NIHem] to measure arterial stiffness, which requires 4 electrodes), and blood pressure cuff on subject.
3. After 20 min, begin blood pressure readings. Perform at least 3, and repeat until measurements are within 5 mmHg, resting 2 min between each reading.
4. Begin tonometry by palpating for the brachial artery pulse and placing the tonometer to record brachial waveforms using the software program.
5. Repeat for the radial, femoral and carotid arteries.

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6. Measure the distance to each of these sites from the supersternal notch using a tape measure (brachial, radial and carotid) and custom ruler/caliper (femoral).
7. Calculate carotid-brachial, carotid-radial, and carotid-femoral (aPWV) using the software program.
8. Place forearm blood pressure cuff just distal to the olecranon process and record at least 10 cardiac cycles of baseline brachial artery ultrasound images and blood flow velocity measurements, with a vascular software set to trigger mode. A mechanical arm can be used to steady the ultrasound probe if desired.
9. Inflate forearm blood pressure cuff to 250 mmHg and begin timer. Instruct participant to remain very still.
10. Begin recording velocities with a vascular software set to trigger mode when the timer reads 4:45. Trigger release the cuff at 5:00 and change the ultrasound to record B-mode (diameter) images when the clock reads 5:10.
11. Continue recording until the clock reads 7:00.
12. Record at least 10 cardiac cycles of baseline brachial artery ultrasound images with a vascular software set to trigger mode.
13. Take subject's blood pressure. If systolic blood pressure $\geq$100 mmHg, place 0.4 mg of sublingual nitroglycerin under the subject's tongue and begin timer, unless the patient has another contraindication.
14. Begin recording B-mode (diameter images) when the clock reads 3:00 with the vascular software set to trigger mode.
15. Stop recording when the clock reads 8:00.
16. Monitor blood pressure until it returns to baseline

4. Preparing Human Umbilical Vein Endothelial Cell (HUVEC) Control Slides

1. Grow HUVECs to passage 5-6 and ~80% confluency.
2. Trypsinize with 3 ml of trypsin or whatever is necessary for the dish/flask.
3. Neutralize trypsin using an equal volume of trypsin neutralizing solution.
4. Centrifuge at 200 x g for ~5 min and remove trypsin and neutralizing solution by vacuum.
5. Resuspend in ~10 ml PBS to wash.
6. Centrifuge at 200 x g ~5 min. Remove PBS.
7. Remove PBS and fix in 1800 µl PBS + 200 µl formaldehyde.
8. Resuspend in PBS (~10 ml).
9. Centrifuge at 200 x g ~5 min. Remove PBS, resuspend in an appropriate volume to add ~200 µl per slide.
10. Store slides at -80 °C until ready for analysis (samples will be fine for many years).

5. Staining of Vascular Endothelial Cells

1. Take slides out of -80 °C freezer and wait 5 min at room temperature (this procedure is for a batch of 10 slides, including one HUVEC control slide).
2. Wipe away excess water with a delicate task wipe (don't touch center of slide).
3. Re-hydrate the slides by adding altered PBS to each slide and leave them for 10 min.
4. While the slides are standing, prepare 5% donkey serum and other solutions.
    1. Prepare 5% donkey serum by adding 300 µl of donkey serum to 5,700 µl of altered PBS (to a pH of 7.4) for up to 10 slides (increase this amount for more).
    2. Dilute the primary antibody of interest in 1,000 µl of 5% serum. For example, nitrotyrosine and NADPH oxidase (1:300 and 1:1,500) could be used as markers of oxidative stress.
    3. Prepare secondary AF568 by diluting 5 µl of AF568 to 1,500 µll of 5% serum.
    4. Prepare VE cadherin by diluting 2 µl of VE Cadherin to 1,000 µl of 5% serum.
    5. Prepare AF488 by diluting 5 µl of VE Cadherin to 1,000 µl of 5% serum.
    6. Keep AF568, VE cadherin and AF488 under foil through the whole process and then place on rocker in 4 °C refrigerator while preparing slides.
5. After 10 min of rehydration, dry slides with a delicate task wipe.
6. Add 5% donkey serum for 60 min and place a piece of plastic paraffin film over the circled area to ensure complete coverage of the chemical.
7. Discard the plastic paraffin film and dry slides with a delicate task wipe. **Do not wash**. Add primary antibody for 60 min and place a piece of plastic paraffin film over the circled area to ensure complete coverage of the chemical.
8. Discard the plastic paraffin film, rinse with altered PBS from squirt bottle and soak in slide columns for 5 min. While the slides are soaking, move them into a dark room. Work in the dark for all remaining steps.
9. Dry slides with Kimwipes, add AF568 (secondary antibody) for 45 min and place a piece of plastic paraffin film over the circled area to ensure complete coverage of the chemical. Cover from light.
10. Discard the plastic paraffin film in the biohazard waste container. Rinse with altered PBS from squirt bottle, and then soak in columns for 5 min.
11. Dry slides with Kimwipes, then add VE Cadherin for 60 min and place a piece of plastic paraffin film over the circled area to ensure complete coverage of the chemical. Cover from light.
12. Discard the plastic paraffin film in the biohazard waste container. Rinse with altered PBS from squirt bottle, and then soak in columns for 5 min.
13. Dry slides with a delicate task wipe, add AF488 for 30 min and place a piece of plastic paraffin film over the circled area to ensure complete coverage of the chemical. Cover from light.
14. Discard the plastic paraffin film in the biohazard waste container. Rinse with altered PBS from squirt bottle, and then soak in columns for 5 min.
15. Dry slides with a delicate task wipe and allow slides to dry for 20 min. Cover from light.
16. Add one drop only of fluoroshield mounting medium with 4', 6-diamidino-2-phenylindole hydrochloride (DAPI) to each slide and cover each with a cover slip.

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17. Place slides in 4 °C refrigerator covered with foil. Imaging needs to be completed within 48 hr.

6. Imaging and Analysis of Vascular Endothelial Cells

1. Prepare microscope for imaging stained endothelial cells according to specifications of the specific microscope. A single blinded technician should analyze any particular protein for a batch of cells.
2. Scan slides systematically. Identify endothelial cells by positive staining for VE Cadherin and confirm nuclear integrity by positive staining for DAPI.
3. Image 30 cells per slide for later analysis. Repeat for each slide in the stained batch, including the HUVEC.
4. Analyze the intensity of the staining for the primary antibody of interest using a qualitative software .
5. To minimize the possible confounding effect of differences in intensity staining between different staining sessions, report values as ratio of protein expression in the collected endothelial cells to the same protein expression in the HUVEC.

Representative Results

FMD_{BA} is quantified as the peak change in diameter of the brachial artery following reactive hyperemia. Thus, the diameter at rest is compared to the diameter following the end of a 5 min blood pressure cuff occlusion period (**Figure 1**). Panel A shows a representative ultrasound image of the brachial artery, and Panel B displays a graph of the R-wave gated change in diameter from cuff release to 2 min following, as obtained using commercially available software. As the change is often quite minimal (In **Figure 1** the change is 4.8%), small differences in measurement can have large impacts on results. Use of commercially available automated edge detection software is highly recommended to minimize bias and potential error in measurement[44,45]. As the stimulus for dilation during reactive hyperemia may differ between groups or conditions being compared, shear rate should be calculated using the Doppler blood flow velocities, and FMD_{BA} should be adjusted for differences when applicable[46,47].

aPWV is calculated with minimal operator input by most commercially available systems, including the NIHem used in our research. The R-wave of the ECG is compared to "foot" of the waveform at a given site and the time difference is calculated (**Figure 2**) for the carotid artery (Panel A) and the femoral artery (Panel B). The distance measurements are used in conjunction with the time differences to calculate a velocity. aPWV refers to velocity between the carotid artery to the femoral artery (*i.e.* along the aorta).

Immunofluorescent analysis of vascular endothelial cells can provide cellular evidence of the level of oxidative stress. To account for differences in staining intensity between staining sessions, the level of fluorescence of a given protein for each individual subject (representative images shown in **Figure 3** Panel A) is compared to the fluorescence of the HUVEC control slide (representative images shown in **Figure 3** Panel B). Thus, differences in protein expression can be compared either between groups or across conditions (*e.g.*, during an intervention study).

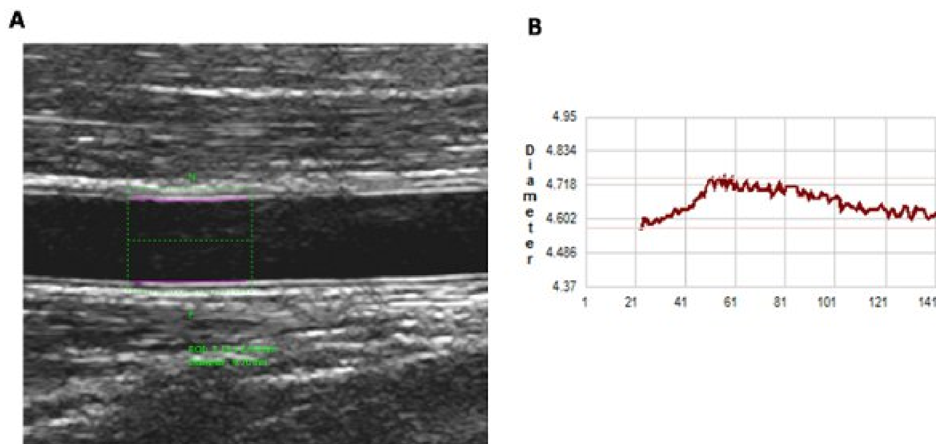

Figure 1. Representative baseline brachial artery diameter obtained during assessment of brachial artery flow-mediated dilation (FMD_{BA}). A) In a patient with chronic kidney disease (CKD). **B)** R-wave gated change in diameter from cuff release to 2 min following is shown graphically, as obtained using commercially available software. Please click here to view a larger version of this figure.

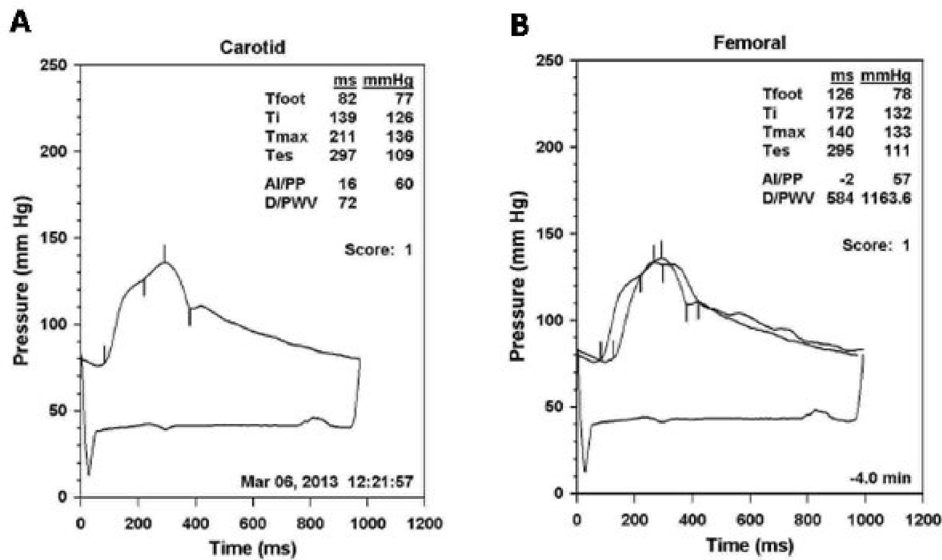

Figure 2. Representative results print-out from assessment of aPWV in a patient with CKD. A) Time delay from the R-wave of the ECG to the foot of the carotid artery, **B)** time delay from the R-wave of the ECG to the foot of the femoral artery (Tfoot), overlaid with the carotid waveform. Both panels also show the inputted distances from the suprasternal notch to the respective sites (represented by the letter D; in cm). The calculated aPWV value is shown in panel B (represented by the letters PWV; in cm/sec). Please click here to view a larger version of this figure.

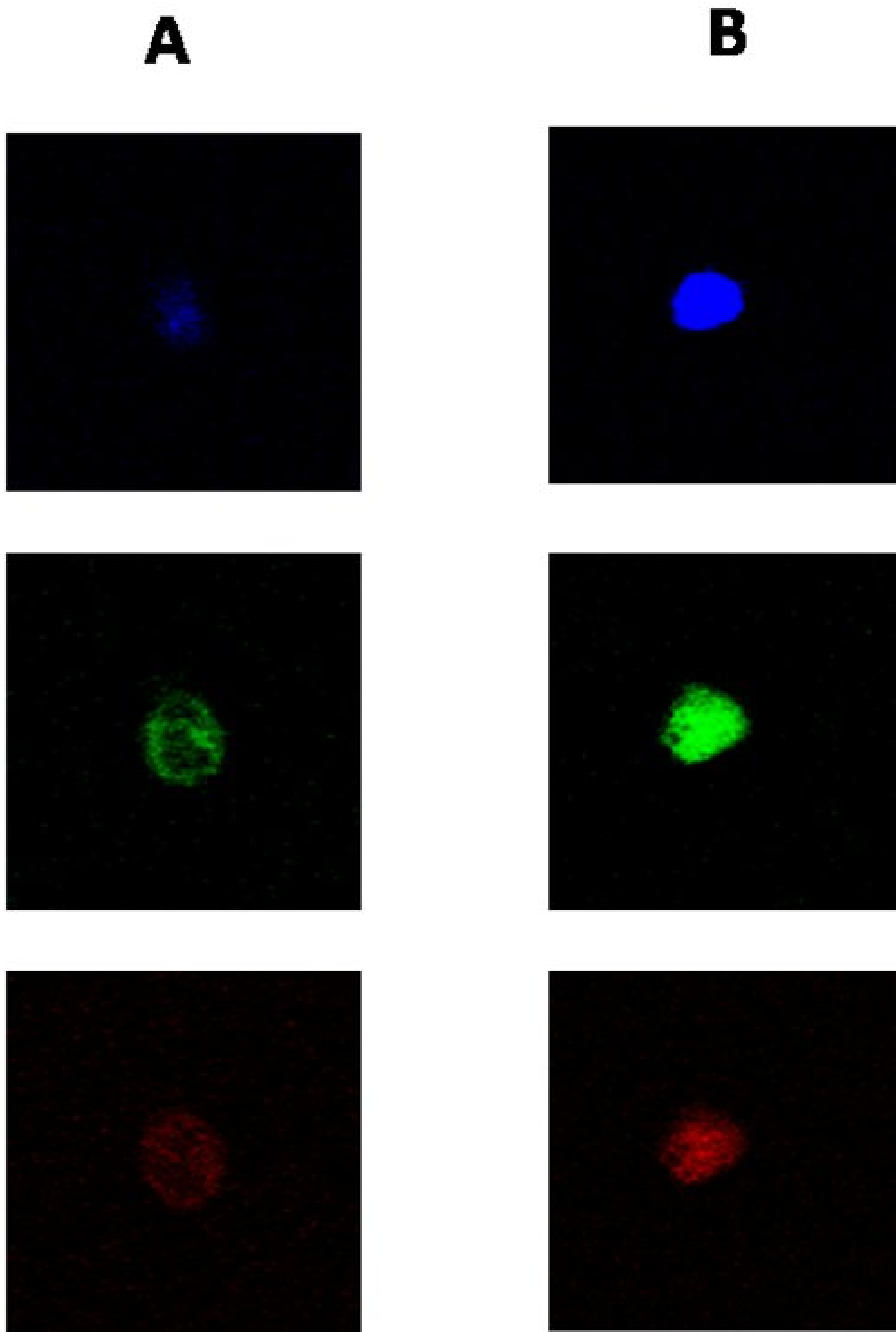

Figure 3. Representative images of protein expression. A) DAPI (nuclear integrity; blue), VE Cadherin (positive endothelial cell identification; green) the oxidant enzyme NADPH oxidase (protein of interest; red) from cells collected from a patient with CKD **B)** and for a human umbilical vein endothelial cell (HUVEC) control slide.

Discussion

Obtaining accurate results for FMD_{BA} and aPWV requires acquiring high quality ultrasound images and pressure waveforms, respectively. Central to this is appropriate and continued training and use of each technique by the operator[44]. In addition, it is critical to control for as many external variables that may influence the results as possible by standardizing the testing session (*e.g.*, prior 12 hr fast, climate controlled room, *etc.*)[44,45]. As mentioned above, the use of commercially R-wave gated acquisition software and edge-detection software is highly recommended to minimize bias and potential error in measurement[44,45]. When FMD_{BA} is impaired, this could be either due to impaired NO release from the

endothelium or due to impaired responsiveness of the vascular smooth muscles to the NO released. Sublingual nitroglycerin is administered to control for the responsiveness of the smooth muscle cell layer to an exogenous nitric oxide donor, in order to conclude that any impairment in FMD_{BA} is specific to the capability of the vascular endothelium to produce nitric oxide[44,45].

As the measurement is a velocity, accurate measurements of both distance and time are critical. The protocol we have described is based on the methodology employed in the Framingham Heart study[24]. Use of raised calipers rather than a tape measure improves accuracy of measurement of distance from the suprasternal notch to the femoral artery by taking a direct path rather than potential measuring over abdominal obesity. A clear "foot" of a clean waveform is absolutely necessary for calculation of the time difference from the R-wave of the ECG to the impulse at the measurement site (see **Figure 2**).

While alternate techniques are available to assess both endothelial function and arterial stiffness, FMD_{BA} and aPWV are both commonly used in clinical research because they are non-invasive and well established as intermediary outcomes. In addition, they are well validated across various populations and are independently predictive of cardiovascular events and mortality[19-26]. Thus, they can be used as surrogate endpoints in clinical studies assessing the efficacy for an intervention to reduce cardiovascular risk in a given population, such as patients with CKD. Modification of these techniques are not required to specifically study patients with CKD, as compared to other populations at risk of CVD.

However, there are important limitations to both FMD_{BA} and aPWV that merit discussion. FMD_{BA} assesses vascular endothelial function of a large conduit artery (the brachial artery), thus does not provide an index of microvascular endothelial function. A separate technique using venous occlusion plethysmography is better suited to assess the latter. However, this methodology requires catheterization of the brachial artery, which is more invasive than FMD_{BA} and may be contraindicated in patients with CKD. In addition, measurement of FMD_{BA} requires lengthy and specific training in order to be performed well. aPWV provides an index of large elastic artery stiffness, which may differ from local arterial stiffness (such as the carotid artery). An alternate technique to assess arterial stiffness is to measure the local arterial compliance (the inverse of stiffness) of the carotid artery, although this is not as widely used or validated with clinical endpoints as aPWV[13]. In addition, the contribution of NO as a determinant of aortic stiffness may vary by vascular bed[48]. Last, there are potential confounds to the interpretation of both FMD_{BA} and aPWV that need be measured and statistically adjusted for as appropriate, including baseline diameter and shear rate for FMD_{BA}[45], and heart rate and blood pressure for aPWV[49].

An important consideration in the collection of vascular endothelial cells is minimizing blood on the J-wires and subsequently on the slides, such that the endothelial cells can be identified with minimal red blood cells overlapping in images. This can be achieved with training for proper technique as well as adequate washing when recovering the cells. When analyzing the slides, it is critical that fluorescence can be objectively quantified and the images are clear, without much background or overlap with other cells. Optimization of dilutions for staining and technique for microscopy analysis prior to analysis of study samples are key steps. Of note, the cell yield of this technique is ~600 vascular endothelial cells per collection, an insufficient amount of total mRNA is available to measure gene expression, thus limiting our probe to immunofluorescent staining of proteins of interest.

In addition to the techniques presented for assessing vascular oxidative stress, circulating or urine markers can be used to assess oxidative stress[12,50]. However, they may be less reflective of the level of oxidative stress specific to level of the vascular endothelium. Using these markers in conjunction with the presented techniques may provide the best indication of the overall level of oxidative stress.

We have provided an overview of methods that can be used to measure FMD_{BA}, aPWV, and vascular endothelial cell protein expression. These techniques are appropriate not only for patients with CKD, but also in other populations at increased risk of cardiovascular disease. Collectively, they provide insight into vascular endothelial dysfunction, large elastic artery stiffness, and contributing physiological mechanisms, including oxidative stress.

Disclosures

The authors declare that they have no competing financial interests.

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