Peripheral Arterial Disease

Multimodality Imaging Approach for Serial Assessment of Regional Changes in Lower Extremity Arteriogenesis and Tissue Perfusion in a Porcine Model of Peripheral Arterial Disease

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Background—A standard quantitative imaging approach to evaluate peripheral arterial disease does not exist. Quantitative tools for evaluating arteriogenesis in vivo are not readily available, and the feasibility of monitoring serial regional changes in lower extremity perfusion has not been examined.

Methods and Results—Serial changes in lower extremity arteriogenesis and muscle perfusion were evaluated after femoral artery occlusion in a porcine model using single photon emission tomography (SPECT)/CT imaging with postmortem validation of in vivo findings using gamma counting, postmortem imaging, and histological analysis. Hybrid \(^{201}\)TI SPECT/CT imaging was performed in pigs (n=8) at baseline, immediately postocclusion, and at 1 and 4 weeks postocclusion. CT imaging was used to identify muscle regions of interest in the ischemic and nonischemic hindlimbs for quantification of regional changes in CT-defined arteriogenesis and quantification of \(^{201}\)TI perfusion. Four weeks postocclusion, postmortem tissue \(^{201}\)TI activity was measured by gamma counting, and immunohistochemistry was performed to assess capillary density. Relative \(^{201}\)TI retention (ischemic/nonischemic) was reduced immediately postocclusion in distal and proximal muscles and remained lower in calf and gluteus muscles 4 weeks later. Analysis of CT angiography revealed collateralization at 4 weeks within proximal muscles (P<0.05). SPECT perfusion correlated with tissue gamma counting at 4 weeks (P=0.01). Increased capillary density was seen within the ischemic calf at 4 weeks (P=0.004).

Conclusions—\(^{201}\)TI SPECT/CT imaging permits serial, regional quantification of arteriogenesis and resting tissue perfusion after limb ischemia. This approach may be effective for detection of disease and monitoring therapy in peripheral arterial disease. (Circ Cardiovasc Imaging. 2014;7:92-99.)

Key Words: perfusion ■ peripheral arterial disease ■ tomography, emission-computed, single-photon
the initial claudication distance (ie, the distance at which pain first occurs), the ankle-brachial pressure index, quality-of-life questionnaires, duplex ultrasonography, and x-ray angiographic parameters. However, ankle-brachial pressure indices can be problematic in the setting of microvascular disease and medial calcification. Ultrasound only permits evaluation of blood flow in major vessels and is not useful for the estimation of collateral vessel flow; and angiography is traditionally used for only anatomic or visual assessment of vessels in the clinical setting. MR imaging can evaluate lower extremity tissue perfusion and oxygenation in the setting of PAD; however, MR techniques are limited by their insensitivity to measure perfusion at rest and usually require exercise or reactive hyperemia protocols to augment flow adequately. Nuclear imaging is capable of detecting abnormalities in lower extremity rest and stress perfusion in the setting of PAD. Additionally, nuclear approaches provide improved sensitivity and, when coredgistered with high-resolution anatomic imaging, offer optimal localization and quantification of radiotracer uptake. Single photon emission tomography (SPECT) imaging with the perfusion radiotracer $^{201}$Tl has proven to be an effective technique for identifying lower extremity perfusion in PAD patients. Additionally, $^{201}$Tl imaging has proven to be a more sensitive approach compared with ankle-brachial pressure index for detecting perfusion abnormalities in asymptomatic patients and may allow for better assessment of microvascular and collateral perfusion compared with standard techniques such as Doppler ultrasound; however, quantitative approaches are still lacking. Quantitative assessment of arteriogenesis, the growth/remodeling of pre-existing arterioles, may also play a critical role in understanding the time course of improvements in PAD patients undergoing therapeutic interventions. However, methods for assessing in vivo arteriogenesis in preclinical models of PAD using x-ray CT or MR angiography have been traditionally limited to visual semiquantitative analysis of collateral vessels, and a majority of studies have quantified arteriogenesis with postmortem angiography and histology. The combined quantitative approaches of SPECT $^{201}$Tl imaging and CT angiography may provide a valuable tool for examining serial responses to therapy and treatment in PAD patients, thereby improving the evaluation and management of this clinical population. Therefore, the purpose of this study was to evaluate serial, regional changes in lower extremity arteriogenesis and resting regional tissue perfusion quantitatively in a clinically relevant porcine model of PAD using in vivo CT angiography and $^{201}$Tl SPECT, with postmortem validation of radiotracer uptake and microvascular density.

Methods

Surgical Preparation

Ischemia was surgically induced in the right hindlimb of 8 male Yorkshire pigs (27.9±5.9 kg) after an overnight fast. Pigs were sedated with oral diazepam (10 mg/kg) and intramuscular ketamine (22 mg/kg). All animals were intubated and mechanically ventilated (Venturi; Cardiopulmonary Corp, Milford, CT) with 35% oxygen, 65% nitrous oxide, and 1% to 3% isoflurane. Blood pressure, oxygen saturation, and an ECG signal were continuously monitored during each imaging session (IntelliVue MP50; Philips Healthcare, Andover, MA). Jugular vein access was established via percutaneous puncture and a 5-F polyethylene catheter was placed for the administration of fluids, CT contrast agent, and radioisotope. The right superficial femoral artery was isolated and 2 ligations, 2.5 cm apart, were placed proximal to the deep femoral artery. All experimental protocols were approved by the Institutional Animal Care and Use Committees at the Yale University School of Medicine and were in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care International policies.

SPECT Imaging

In vivo, single-isotope $^{201}$Tl SPECT imaging was performed at baseline, immediately postocclusion, and at 1 and 4 weeks after femoral artery occlusion. Imaging was performed 15 minutes after intravenous injection of $^{201}$Tl (99.9±12.4 MBq) at rest. All images were acquired with a dual-headed camera capable of CT attenuation (Millenium VG; GE Healthcare) using a 360° step and shoot acquisition with a 78 keV±10% window, 3° projections, and 30 seconds per stop. Immediately after the SPECT acquisition, CT images were acquired and reconstructed with a filtered back projection to create CT attenuation maps. CT-based attenuation correction was applied for SPECT image reconstruction using 2 iterations and 10 subsets of the ordered subset expectation and maximization algorithm. SPECT images were smoothed using a 3-dimensional (3D) Butterworth filter with a cutoff frequency of 0.4 cycles/cm and an order of 10 (Xeleris Workstation; GE Healthcare).

CT Angiography

In vivo CT angiography was performed at baseline, immediately postocclusion, and at 1 and 4 weeks postocclusion using a 64-slice CT scanner (Discovery NM-CT 570c; GE Healthcare) with iodinated contrast (300 mgI/mL; Omnipaque, GE Healthcare). Images were acquired at a slice thickness of 0.625 mm, at 300 mA, and 120 kVp. Intravenous contrast injections were performed with a power injector (Stellant D; MEDRAD, Warrendale, PA) at a constant rate of 3 mL/s and total volume of 30 mL, followed by a 0.9% normal saline flush at 3 mL/s.

Segmentation of Region of Interest

3D muscle regions of interest (ROI) were manually drawn in the ischemic and nonischemic hindlimbs from contrast CT angiograms (Figure 1). All image segmentation and analysis was performed with BioImage Suite (http://www.bioimagesuite.org), an image analysis toolkit developed at Yale University. No differences in ROI volumes...
were found across time for any of the segmented muscle regions (P>0.05), indicating that anatomic ROIs were similarly segmented for each time point.

Quantification of Regional Microvascular Perfusion
Average retention of 201Tl within the ischemic and nonischemic hindlimbs was quantitatively assessed in segmented 3D muscle ROIs. All values for relative 201Tl perfusion are expressed as ischemic/non-ischemic ratios.

Quantification of Regional Arteriogenesis
3D volumetric analysis of CT contrast within selected muscle groups was performed to quantify serial changes in arteriogenesis within each ROI. First, average intensity and SD values from analysis of CT angiograms (contrast within soft tissues) were recorded. Soft tissue was then removed by image thresholding so that only contrast within the vasculature remained. Threshold levels were set at 1.5 SD above the average image intensity value for a given muscle ROI. The remaining 3D contrast volume was quantitatively assessed within each ROI. All values for quantification of intramuscular arteriogenesis are expressed as ischemic/nonischemic ratios.

Postmortem Gamma Counting
After euthanization at 4 weeks postocclusion, multiple tissue samples weighing ≈1 g each were extracted from ROIs within the ischemic and nonischemic hindlimbs to measure 201Tl radioactivity with a gamma counter (Cobra 5003; Packard Instrument Co, Meriden, CT). Care was taken to ensure that tissue was sampled from similar anatomic locations within each muscle region of both hindlimbs and that each muscle sample was absent of subcutaneous fat. All values for 201Tl retention were calculated as a percent of the injected dose per gram of tissue (after correction for background activity and radioactive decay) and also expressed as ischemic/nonischemic ratios.

Postmortem Capillary Density
Capillary density was quantified in 4 animals in the gastrocnemius and semimembranosus muscle groups of the ischemic and nonischemic hindlimbs on 5-μm thick muscle sections (fixed in 4% paraformaldehyde and embedded in paraffin) that were stained with isolectin B4 (IB4; Sigma-Aldrich, St Louis, MO) using techniques previously reported.31 Values are expressed as capillary/fiber ratios. Suitable cross-sections for analysis contained nearly circular fibers and clear round-shaped capillaries. Quantification was performed in images taken at ×20 magnification using a minimum of 3 images per muscle (Eclipse 80i; Nikon Instruments Inc, Melville, NY). Care was taken to ensure that tissue was sampled from similar anatomic locations within each muscle region.

Postmortem CT Angiography
Sixty-four-slice x-ray CT angiography (Discovery NM-CT 570c; GE Healthcare) was performed after the casting of lower extremity vessels to enhance visualization of small vessels and validate the presence of collateral formation (n=4). Before euthanization, each animal was administered 3 mL of heparin (1000 IU/L) intravenously. After euthanization, the abdominal aorta was cannulated and the vasculature was flushed with 0.9% normal saline. Approximately 230 mL of contrast agent containing 15% bismuth (Sigma-Aldrich) in 10% gelatin was then delivered by hand injection. Images were acquired at a slice thickness of 0.625 mm, at 300 mA, and 120 kVp. Commercially available software (AW workstation v4.4; GE Healthcare) was used to reconstruct 3D images, and bone was removed from images via manual segmentation. H&E staining was performed on gastrocnemius muscle tissue to validate the presence of bismuth contrast within the microcirculation of the distal hindlimb. Muscle samples were cut transversally, fixed in 4% paraformaldehyde, paraffin embedded, and serial sectioned (5 μm sections). Images were obtained at ×10 and ×20 magnification.

Statistical Analysis
A repeated measures univariate mixed model was used to identify differences in serial assessment of CT angiography and SPECT perfusion. This mixed model approach permitted serial assessment of the CT angiograms and SPECT perfusion scans while allowing for inclusion of data for 3 animals that had missing data for some imaging time points of the study (images were available for 27 of 32 potential serial images from the 8 animals). To assess the relationship between in vivo SPECT image analysis and postmortem gamma counting of tissue at 4 weeks after femoral occlusion, a multilevel linear model was used that controlled for the nonindependence of the analyzed muscle ROIs. Capillary density analysis was performed using Student t test. All statistical analyses were performed using commercially available software (IBM SPSS Statistics for Windows, v19.0; IBM Corp, Armonk, NY). Statistical significance for all analyses was set at P<0.05. All values are expressed as mean±SD unless stated otherwise.

Results
Analysis of Microvascular Perfusion
Regional tissue perfusion was significantly impaired within the calf muscles of the distal ischemic leg immediately after occlusion (baseline, 1.04±0.08; acute occlusion, 0.71±0.05; P=0.001) and remained lower for 4 weeks (Figure 2A and 2B). 201Tl perfusion was similarly reduced within the glu- teus muscle region of the ischemic limb after occlusion (P=0.0001) and remained reduced at 1 (P=0.0001) and 4 (P=0.01) weeks postocclusion (Figure 2B). Perfusion to the biceps femoris, semimembranosus, and semitendinosus regions was significantly reduced immediately after and 1 week postocclusion, but relative 201Tl perfusion normalized within all of these regions of the proximal limb by 4 weeks (Figure 2B).

Analysis of Arteriogenesis
Arteriogenesis was qualitatively assessed from CT angiograms at 4 weeks postocclusion (Figure 3A). Qualitative observations were confirmed through quantitative image analysis of 3D contrast volume within hindlimb muscle regions, based on defined CT image thresholding limits (Figure 3B). Specifically, significant increases in relative contrast volume (ischemic/nonischemic) were found in the biceps femoris (baseline, 0.97±0.06; 4 weeks, 1.07±0.05; P=0.001) and semimembranosus (baseline, 0.98±0.04; 4 weeks, 1.06±0.05; P=0.001) muscle regions of the proximal hindlimb. No significant differences across time were observed in the calf, gluteus, or semitendinosus muscle regions (Figure 3B).

Postmortem Gamma Counting
Postmortem gamma well counting of muscle tissue at 4 weeks postocclusion was significantly and positively related to in vivo SPECT image analysis when entered into a multilevel model that controlled for the nonindependence of muscle ROIs (F[1,19.49]=7.50; P=0.01; β=0.48; Wald=2.74; P=0.01; Figure 4).

Histological Analysis of Angiogenesis
Histological analyses revealed significant differences in relative capillary density (expressed as capillary/fiber ratios) between ischemic and nonischemic hindlimbs within the gastrocnemius muscle of the distal hindlimb (ischemic: 1.32±0.40; nonischemic: 0.95±0.10; P=0.004; Figure 5). No
difference was observed in the semimembranosus muscle of the proximal hindlimb (ischemic: 0.97±0.10; nonischemic: 1.02±0.13; P=0.32).

Postmortem Validation of Arteriogenesis
Postmortem CT angiography confirmed the presence of collateral vessels in the ischemic hindlimb and provided

Figure 2. Serial quantification of in vivo single photon emission tomography (SPECT)/CT imaging. A, ²⁰¹Tl SPECT imaging revealed visible resting perfusion deficits in the muscles of the proximal (white arrow, top row) and distal hindlimb on the day of femoral artery occlusion. A progressive increase in perfusion is seen at 1 and 4 weeks after occlusion. B, SPECT image analysis revealed significant perfusion defects in all muscle regions, which gradually improved after 4 weeks. I indicates ischemic hindlimb; and NI, nonischemic hindlimb. N=8; †P<0.05; ††P≤0.01; ‡P=0.0001.

Figure 3. Regional quantification of in vivo CT angiography. A, Serial CT angiograms are shown for a representative animal. 3-Dimensional CT angiography visually confirmed successful ligation of the femoral artery (white arrow) and collateral vessel formation at 4 weeks postocclusion (yellow arrow). B, Vessel volume was significantly higher from baseline in the semimembranosus and biceps femoris muscle regions after 4 weeks of occlusion. N=8; †P<0.01.
Quantification of 201Tl SPECT and CT angiography offers a combined in vivo image analysis approach with regional calf muscles, capillary density was significantly increased although perfusion levels were not completely restored in the gamma counting at the 4-week time point. Additionally, findings by SPECT image analysis and validated by postmortem improved visualization of the microcirculation and collateralization at 4 weeks postocclusion compared with in vivo contrast CT angiography (Figure 6A and 6B). H&E staining confirmed the penetration of bismuth contrast into the microcirculation of the gastrocnemius muscle of the hindlimb (Figure 6C).

**Discussion**

In the present study, we demonstrate the feasibility of regional, quantitative assessment of serial changes in tissue perfusion and arteriogenesis in the presence of lower extremity ischemia using 201Tl SPECT and CT angiography. We found significant regional reductions in relative tissue perfusion with 201Tl SPECT immediately after unilateral femoral artery occlusion in a porcine model of PAD. Recovery of perfusion to baseline levels occurred in a majority of muscle ROIs of the proximal hindlimb by 4 weeks after occlusion; however, perfusion remained significantly reduced in the gluteus and calf muscles at 4 weeks (Figure 2A). The observed serial changes in tissue perfusion may be at least partially attributed to significant arteriogenesis that was observed 4 weeks after occlusion in the proximal hindlimb (Figure 3). Arteriogenesis within the semimembranosus and biceps femoris muscle regions presumably resulted in improved downstream tissue perfusion, which was quantified by SPECT image analysis and validated by postmortem gamma counting at the 4-week time point. Additionally, although perfusion levels were not completely restored in the calf muscles, capillary density was significantly increased within the gastrocnemius 4 weeks after occlusion (Figure 5). A combined in vivo image analysis approach with regional quantification of 201Tl SPECT and CT angiography offers potential benefit for the noninvasive, serial evaluation of PAD patients undergoing various forms of treatment, including exercise programs, medications, revascularization, and novel gene or cell therapies.

CT angiography, which provides visualization of vessel morphology, is a common technique for assessing the extent of PAD and provides guidance for vascular interventions. Although visual analysis of in vivo contrast angiograms allows for qualitative assessment of vascular remodeling, the quantitative approach used in the present study for the evaluation of arteriogenesis allows for objective assessment of vascular remodeling in specific muscle regions of the lower extremities after intervention. Previous studies have used semiquantitative analysis of arteriogenesis in preclinical models of PAD. Analyses in most of these studies were limited to subjective in vivo analysis of collateral formation or postmortem angiographic and histological evaluation. In our study, we sought to develop a standardized CT image analysis approach for quantification of in vivo arteriogenesis in a large animal model of PAD that could be directly translated to patients. All images were independently thresholded based on relative intensity values of soft tissue and CT contrast to allow for regional assessment of 3D contrast volume (arteriogenesis) at multiple time points. The threshold level (average intensity +1.5 SD) was determined based on visualization of the most optimal contrast-to-soft tissue ratio. Our image analysis approach revealed significant vascular remodeling that occurred within the medial (semimembranosus) and lateral (biceps femoris) muscle regions of the proximal hindlimb after femoral artery occlusion (Figure 3).

SPECT imaging with 201Tl allows for evaluation of microvascular as well as collateral perfusion at rest. In the present study, we found significant decreases in relative regional microvascular perfusion resulting from arterial occlusion, with the most pronounced changes occurring within the calf muscles (baseline, 1.04±0.08; acute occlusion, 0.72±0.05). These results are in agreement with a study by Earnshaw et al that found similar relative decreases in 201Tl perfusion within the calves of patients with iliac artery occlusion or stenosis. We also observed significant changes in tissue perfusion within all other muscle ROIs, with a majority of muscles recovering to baseline levels by 4 weeks after occlusion. Postmortem gamma counting of muscle tissue samples at 4 weeks correlated with SPECT image analysis, confirming the existence of regional differences in 201Tl uptake and providing a validation for our quantitative, noninvasive approach for assessment of muscle perfusion. Significant microvascular remodeling was observed by immunostaining in the gastrocnemius muscle (within the calf muscle region; Figure 5). This may partially explain serial improvements in lower extremity perfusion although complete recovery of microvascular perfusion in the distal calf muscles did not occur within 4 weeks of arterial occlusion. These results suggest that noninvasively quantifying regional changes in resting tissue perfusion with SPECT/CT may be useful for evaluating specific muscle groups that are susceptible to impaired perfusion in the setting of PAD, both for diagnosis and evaluating response to treatment.

**Limitations**

Although our image thresholding approach seems to be useful for large- and intermediate-sized vessels, in vivo assessment of smaller vessels (<500 µm) with this technique is not possible.
Image thresholding of in vivo CT angiography was unable to differentiate small vessels from soft tissue with varying threshold levels. Therefore, our method may only be sensitive enough to detect large collateral vessels, whereas smaller collaterals may be eliminated via thresholding. Multiple threshold levels were attempted before determining the parameters for CT contrast image analysis. This was based on a binary visualization of vascular structures, without inclusion of soft tissue.

Figure 5. Assessment of capillary density. Representative cross-sectional images (x20 magnification) of ischemic and nonischemic gastrocnemius (A) and semimembranosus (B) muscle stained with isolecitin B4. A significant increase in capillary density was seen at 4 weeks postocclusion in the gastrocnemius, whereas no difference was observed in the semimembranosus muscle. Bars represent mean±SD. N=4.

Figure 6. Postmortem CT angiography. A, In vivo CT angiography revealed collateral development at 4 weeks after femoral artery occlusion (white arrow). B, Postmortem angiography with bismuth gelatin allowed for improved visualization of the microcirculation in both hind limbs. C, H&E staining of the gastrocnemius muscle confirmed the presence of bismuth contrast at various microscopic levels in the microcirculation (black arrows).
201Tl SPECT studies were performed only at rest. This approach could be easily performed during both rest and stress conditions in patients. Although 201Tl demonstrates a component of redistribution, we performed imaging at 15 minutes postradiotracer injection to minimize any redistribution. To further evaluate and make more easily integrated with rest and stress myocardial perfusion imaging, Future studies comparing regional differences in perfusion and arteriogenesis under both rest and stress conditions are warranted.

Conclusions
In vivo quantitative assessment of integrated CT angiography and SPECT imaging is able to distinguish regional, time-dependent changes in arteriogenesis and tissue perfusion at rest. The unique ability to evaluate regional ischemia in the lower extremities under resting conditions is of special importance, given the lifestyle-limiting claudication experienced by many PAD patients. Combined quantitative SPECT/CT imaging may offer a unique opportunity for examining serial responses to therapy in patients with PAD. Future studies could integrate this evaluation of PAD with exercise or pharmacological stress imaging of the heart and may offer the opportunity for the assessment of vascular reserve.

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Disclosures
None.

References
Peripheral arterial disease (PAD) is a highly prevalent, progressive atherosclerotic disease of the lower limbs affecting more than 8 million Americans. Assessment of therapy in PAD patients is crucial given the significant impact of this disease on life expectancy, functional status, and quality of life. Standard quantitative imaging tools for serial evaluation of lower extremity arteriogenesis and tissue perfusion are not readily available. The availability of quantitative image analysis tools at both the microvascular and macrovascular levels could allow for noninvasive serial assessment of therapy in patients with PAD. The present study demonstrates the feasibility of quantifying serial changes in lower extremity arteriogenesis and microvascular perfusion at rest within specific muscle groups using CT angiograms and 201Tl single photon emission tomography (SPECT) in a large animal model of PAD. Our imaging results were validated with postmortem tissue analyses. Progressive improvement of both muscle perfusion and collateralization was demonstrated in the ischemic lower extremities, in association with significant differences in capillary density, suggesting that downstream improvements in perfusion may be attributed to adaptations occurring at the micro- and macrovascular levels within 4 weeks after femoral artery occlusion. This integrated 201Tl SPECT/CT and CT angiography imaging approach for quantifying regional changes in microvascular perfusion and collateralization can be applied for evaluating the response to medical or surgical therapeutic interventions in PAD patients. These techniques can be directly translated into patients with lower extremity disease who are undergoing clinically indicated myocardial perfusion rest/stress imaging for preoperative risk stratification with little additional cost or risk.
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