Noninvasive Assessment of Foot Perfusion in a Rabbit Model of Atherosclerosis Using Dynamic Volume Perfusion CT with an Upslope Method

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Abstract

Objectives: To evaluate the feasibility of foot dynamic volume CT with the upslope method and to demonstrate macrovascular reactivity and microvascular perfusion during cuff-induced reactive hyperemia state in a rabbit model of atherosclerosis.

Materials and Methods: 30 New Zealand male rabbits were divided into 2 groups: dietary hypercholesterolemia induced atherosclerosis (n=10) and normal diet control (n=20). To measure for macrovascular reactivity, perfusion parameters of the left posterior tibial artery was measured at baseline and at reactive hyperemia state. For the evaluation of microvascular perfusion, color-coded perfusion map of the plantar dermis was generated for perfusion CT scan by an in-house dedicated analysis software based on upslope method. Dermal perfusion values were measured and analyzed before and after cuff-induced reactive hyperemia.

Results: Foot dynamic volume CT with the upslope method demonstrated significant impairment of both macrovascular reactivity and microvascular perfusion in atherosclerotic rabbits during cuff-induced reactive hyperemia (CRH) state. Arterial time-to-peak of cholesterol-fed rabbits failed to show acceleration while healthy rabbits showed significant decrease in time. Microvascular perfusion calculated by perfusion value ($P<0.01$) and perfusion ratio ($P=.014$) showed decreased microvascular perfusion in cholesterol fed rabbits compared to healthy rabbits during CRH state. Post-CT pathologic examination revealed decreased endothelial cell density in cholesterol-fed rabbits ($P<0.001$).

Conclusions: Foot perfusion CT using upslope method provides perfusion parameters for large arteries and a perfusion map of the foot during cuff-induced reactive hyperemia in rabbit models of atherosclerosis. It may be a useful tool for the quantitative and functional evaluation of atherosclerotic peripheral arterial disease.

Summary Statement

Dynamic volume CT with upslope method provides a perfusion map of the foot and reveals impairment during reactive hyperemia in a rabbit model of atherosclerosis.

Key Results

- Dynamic volume CT with upslope method was used to generate a perfusion map to evaluate macrovascular reactivity and microvascular perfusion in a rabbit model of atherosclerosis.
- During cuff-induced reactive hyperemia state, rabbit models of atherosclerosis showed a higher arterial time-to-peak in its tibial artery compared to healthy rabbits, suggesting deterred vasoreactivity of large arteries.
- Compared to healthy rabbits, atherosclerosis group showed impaired perfusion in the foot during cuff-induced reactive hyperemia state.

Introduction

Atherosclerotic peripheral arterial disease (PAD) is a global health problem, afflicting over 200 million people worldwide ¹⁻³. PAD is the third leading cause of atherosclerotic cardiovascular morbidity, following coronary artery disease and stroke ¹. Disability and mortality associated with PAD has significantly increased over the last 20 years ⁴.

Ankle brachial index (ABI) is the most readily available, and validated method to screen and diagnose PAD ⁵. ABI relies on macroscopic measurements of pressure gradient in the limbs to provide important diagnostic and prognostic information, such as risk of death, myocardial infarction and stroke ^{6,7}. Yet, despite the diagnostic and prognostic utility, ABI does not give any anatomical information. It is also not applicable to evaluate non-compressible arteries with heavy calcification or below-ankle diseases ^{8,9}. Other modalities such as peripheral artery tonometry (PAT) or flow mediated dilatation (FMD), while limited in use, can evaluate macrovascular reactivity but do not provide any anatomical information. Modalities that provide anatomic information for the evaluation of PAD such as nuclear imaging, computed tomographic (CT) angiography, magnetic resonance (MR) angiography, and conventional angiography visualize macrovascular lesions but do not evaluate microvascular perfusion ¹⁰⁻¹². The ideal test for PAD would provide both macrovascular and microvascular information as well as anatomical details to assess disease severity, guide decision-making, and predict treatment response. Therefore, a pragmatic modality to concurrently evaluate arterial blood flow, microvascular foot perfusion and anatomic lesions is imperative.

Perfusion imaging has been widely used to diagnose and treat coronary artery diseases and cerebral ischemic diseases ¹³⁻¹⁵. In evaluating cerebral ischemic diseases, perfusion CT or MRI allows for the visualization of ischemic penumbra zones, providing practical information on brain tissue viability ^{13,14}. In the evaluation of coronary artery disease, the concept of “myocardial perfusion reserve” allows for the evaluation of the surrounding microvasculature’s ability to meet tissue demands upon stressors ¹⁶. Perfusion reserve, defined as the ratio of global blood flow at stress vs. rest, allows for identification of at-risk tissue. Widely used in the clinic, stress testing with adenosine can induce vasodilatation and hyperemia which allows for the identification of myocardial tissue with hypoperfusion in asymptomatic individuals without abnormalities on conventional angiography ¹⁶. These imaging modalities provide important information to non-invasively identify areas at risk and guide treatment. However, perfusion imaging modalities have yet to be applied for the evaluation of the foot for patients with atherosclerosis or claudication but no significant artery occlusion.

In our previous study, we showed that foot perfusion CT with the upslope method can provide both qualitative and quantitative assessment of the foot in patients with PAD ¹⁷. In addition, we identified the presence of a dermal hyperperfusion band in the rabbit’s feet that served as the region of interest in evaluating foot tissue perfusion¹⁷.

Here, we evaluated the feasibility of dynamic volume CT with the upslope method during cuff-induced hyperemia, to demonstrate macrovascular reactivity and microvascular perfusion in a rabbit model of atherosclerosis.

Materials And Methods

Statement

All experiments and methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by a named institutional/licensing committee. Specifically, animal research protocol was approved by Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital. The study was carried out in compliance with the ARRIVE guidelines. All animals were euthanized with an intravenous injection of a lethal amount (7-10 ml) of xylazine hydrochloride under deep anesthesia.

Animal Model Preparation

Thirty New Zealand white male rabbits weighting 3000-3500 g were used in our study. All animals were housed in cages with a 12-hour, light/dark cycle and *ad libitum* access to water. For anesthesia, 5 mg/kg body weight tiletamine-zolazepam (Zoletil 50; Virbac, Carros, France) and 2 mg/kg body weight of 2% xylazine hydrochloride (Rompun; Bayer, Seoul, Republic of Korea) were injected intramuscularly in the posterior thigh. Atherosclerosis was induced as described in previous studies ¹⁸⁻²². 10 rabbits in the atherosclerosis group were fed an atherogenic diet consisting of 0.5% cholesterol and 6% peanut oil for 5 weeks and then switched to a diet containing lower cholesterol (0.025%) for another 5 weeks to prevent liver failure ^{18,22}. The 20 rabbits in the control group (group B) were fed normal rabbit chow for all study periods. Study protocol for induction of atherosclerosis is schematically shown in Figure 1.

Angiography

All rabbits underwent angiography to evaluate the presence of significant stenosis or occlusion of bilateral aortoiliac and femorotibial arteries, one day prior to CT scan. After sedation, rabbits were placed in supine position and right central ear artery of each rabbit was cannulated using 16 gauge IV cannula. To access the infrarenal aorta, a 2.0-Fr microcatheter (progreat a, Terumo, Tokyo, Japan) was advanced via the ear artery to the iliofemoral artery and femorotibial angiography was performed.

Foot Perfusion CT

Perfusion CT protocol similar to previous experiment was performed to evaluate foot perfusion status of each rabbit as detailed in a previous study ^{17,17}. In brief, each animal was laid down on the table of a 64-

detector CT scanner (Ingenuity; Philips Medical, Eindhoven, the Netherlands) with 4-cm z-axis coverage in volume scan mode. First, baseline perfusion CT scan was obtained by using following parameters: scan coverage including the whole hindfoot and ankle, volume scan mode without table movement, gantry rotation time of 400 msec, detector collimation of 64 x 0.625 mm, tube voltage of 100 kV, and tube current of 100 mA. The dynamic volume scanning started 1–3 seconds after intravenous bolus injection of 3mL of iopamidol (Pamiray, 370mg of iodine per milliliter; Dongkook Pharmaceutical, Seoul, South Korea) contrast material. Contrast material injection was followed by injection of an equal volume of normal saline, both injected with an automated dual-rail injector (Stellant; Medrad, Warrendale, Pa) at a rate of 1 mL/sec via the left auricular vein. After 5 minutes of rest, a disposable neonatal blood pressure cuff (Welch Allyn, NY, USA) was applied to animal's left thigh and cuff was inflated to 200 mmHg to block blood flow to left foot for 3 minutes. As the cuff deflated to 0 mmHg within 3 seconds, a second perfusion CT was obtained in the same manner as the previous scan²³⁻²⁶. Perfusion CT protocol timeline was illustrated in Figure 1.

Image reconstruction and analysis of perfusion CT findings

All the CT images were transferred to an image archiving and communications workstation (INFINITT; Infinitt healthcare, Seoul, Republic of Korea) and analyzed by in-house perfusion analysis software¹⁷. The image reconstruction method for the perfusion CT data was identical to that of a prior animal experiment¹⁷, except that the plantar dermis in the heel base rather than that of the toes was used as the representative tissue to set the upslope time range. Each session of perfusion CT data was sent to the software and a pixel-based, color-coded perfusion map was generated by using the upslope method¹⁷. Based on a perfusion map, region of interest (ROI) for posterior tibial artery and plantar dermis was placed, and time-attenuation curve¹ was calculated respectively (Fig 1). Upslope time range was set from the arterial time-attenuation curve and the following perfusion parameters were obtained; Peak arterial enhancement (PAE), Time to peak enhancement (TTP), and maximal upslope (m). Time attenuation curve of each pixel was normalized by the maximal arterial enhancement value. Quantitative analysis of perfusion parameters was performed by two radiologists (S.H and H.D.M) in consensus. Regional blood flow at baseline perfusion CT scan was measured at the dermis of the foot sole by placing an ROI on the axial image. In the same way, ROI was set in the corresponding location of the plantar dermis on the perfusion map obtained from the second CT scan, and each perfusion value and the ratio before and after inducing CRH was calculated.

Pathological analysis

All animals were preanesthetized and sacrificed with an intravenous injection of xylazine hydrochloride. Dermal tissues of left heel of all rabbits were harvested. Tissues obtained were weighed and half of the tissues were processed to evaluate number of each color's microsphere, the other specimens were fixed in 10 % buffered formalin and were embedded in paraffin. Thereafter, tissue samples were treated with

hematoxylin and eosin staining for basic histopathological examinations and consecutive section was treated CD 31 staining (DAKO Corp., Carpinteria, CA, USA) for microvascular density (MVD) evaluation. After digital images of the histologic slides were obtained (Leica Microsystems, Mannheim, Germany), MVD was calculated using image analysis software (Image J, version 1.45s; National Institutes of Health, Bethesda, MD) (11). The five hot spots with the most intense vascularization were selected by L.J.H who was blinded to information of animal group, while screening at a low-power field ($\times 40$). MVD counts of the five areas per the hot spots were performed at a high-power field (S.B.C) ($\times 100$). Any brown-stained endothelial cells or endothelial cell clusters clearly separated from adjacent microvessels were counted as one microvessel stained by the anti-CD31 antibody, irrespective of the presence of a vessel lumen. The mean microvessel area percentage of all the measured areas was determined to be the MVD.

Statistical analysis

All data in the study was reported as mean $\pm$ SD. The perfusion value and ratio of regional blood flow between baseline and CRH in each group was tested by using paired-T test and Wilcoxon signed rank test. The value of change of each parameter between two groups were compared using repeated measures analysis of variance test. The data processing and analysis were performed using Statistical Package for the Social Sciences version 18.0 (SPSS Inc, IBM Company, Chicago, IL). A two-sided P value of less than 0.05 indicates that the groups differ significantly in terms of statistical results.

Results

Angiography and Foot perfusion CT

Angiography was performed successfully in all rabbits, and there were no significant stenosis or occlusions in the aortoiliac and femorotibial arteries in both groups (Fig 2). Foot perfusion CT was successfully performed in all rabbits from both groups at baseline and after cuff-induced reactive hyperemia (CRH) state.

Quantitative analysis of arterial perfusion parameters at baseline and cuff-induced reactive hyperemia

Serial measurements of arterial perfusion parameters were taken at baseline and CRH phase in both the healthy and the cholesterol-fed groups. At baseline, cholesterol-fed rabbits compared to healthy rabbits showed increased PAE, increased maximum arterial upslope, m but no differences in TTP (cholesterol-fed vs healthy rabbits; 354.6 ± 96.47 HU vs 255.51 ± 96.22 HU, $P < 0.001$; 109.30 ± 25 vs 57.35 ± 33.44 HU, $P < 0.001$; 10.7 ± 1.82 HU vs 11.65 ± 2.32 HU, $P = 0.269$, respectively) (Fig 3). During CRH phase, both groups showed an increase in PAE and m compared to baseline, but intergroup differences were not significant ($P = .922$ and $P = .977$, respectively) (Fig 3). However, the arterial TTP of the healthy group showed

significant acceleration, whereas that of cholesterol-fed rabbits failed to show a surge (11.65 ± 2.32 sec to 9.80 ± 1.76 sec for healthy rabbits, 10.70 ± 1.82 to 10.50 ± 1.35 for cholesterol-fed rabbits, $P = .025$) (Fig 3).

Dermal perfusion value and perfusion ratio analysis between baseline and cuff-induced reactive hyperemia

Figure 4 summarizes the result of perfusion values of left plantar dermis in the two groups at baseline and CRH phase. Perfusion values were calculated as PAE multiplied by heel-base perfusion. There was no significant difference of dermal perfusion value between healthy group and cholesterol-fed group at baseline (35040.61 ± 17750.58 vs. 47427.59 ± 18968.08 ; $P = 0.57$) (Fig 4a). Dermal perfusion value of healthy rabbits significantly increased on CRH phase (35040.61 ± 17750.58 to 57582.43 ± 22585.11 HU; $P = 0.008$), whereas no such surge was demonstrated in cholesterol-fed groups (47427.59 ± 18968.08 to 46692.38 ± 19797.93 HU; $P = 0.878$) (Fig 4a). The perfusion ratio (dividing perfusion values of CRH to that of baseline) of left foot for normal rabbits were significantly higher than that of cholesterol-fed rabbits (1.62 ± 0.74 vs. 0.99 ± 0.18 ; $P = 0.014$) (Fig 4b).

Endothelial cell density between healthy and cholesterol-fed groups

All animals were sacrificed post CT scans and foot tissue was pathologically examined for differences in endothelial cell density. Endothelial cell density as measured by CD 31 showed decreased endothelial density in cholesterol fed rabbits (2.22 ± 0.24 vs. 0.69 ± 0.04 ; $P < 0.001$) (Fig 5.).

Discussion

The present study showed that the combination of dynamic volume perfusion foot CT with the upslope method and cuff-induced reactive hyperemia (CRH) was a feasible method for evaluating macrovascular reactivity and microvascular perfusion in a rabbit model of atherosclerosis. Using this combination, impairment of macrovascular reactivity and microvascular perfusion of the cholesterol-fed rabbits were non-invasively and quantitatively demonstrated.

In this study, cholesterol-fed rabbits compared to healthy rabbits showed increased PAE, increased maximum arterial upslope, m but no differences in TTP. However, during CRH phase, the atherosclerosis rabbits showed a hindered vasoreactivity as implied by the delayed arterial TTP. A delayed TTP on reactive hyperemia state implies that the target artery has lost appropriate reactivity to hyperemia leading to hypoperfusion. As atherosclerotic burden progresses, arterial TTP decreases reflecting disease severity^{27–30}. Our previous study showed that decreased arterial TTP of patients with PAD was improved after revascularization therapy¹⁷.

Perfusion value (peak arterial enhancement multiplied by heel based perfusion), a surrogate measure of autoregulation, increased during CRH phase compared to baseline for healthy rabbits. However, this autoregulation did not occur in the atherosclerotic rabbits. We hypothesized that the lack of autoregulation without any significant stenosis or occlusions in the atherosclerotic rabbits was due to endothelium dysfunction. Previous studies show that endothelium dysfunction occurs even before any apparent structural changes to vessel walls are detected on angiography or ultrasound³¹. Studies with acetylcholine test or measurements of FMD detected endothelial dysfunction at both conduit and microvascular levels in patients with coronary risk factors but no structural evidence on angiography or ultrasound^{32,33}. Our study confirmed that cholesterol-fed rabbits had a lower endothelial cell density, suggesting possible endothelium dysfunction as mechanism for impaired autoregulation during CRH phase.

Foot perfusion ratio decreased in the atherosclerotic rabbit compared to those of healthy rabbits. As perfusion ratio reflects surrounding microvasculature's ability to meet tissue demands upon hyperemia, combined dynamic foot perfusion CT with CRH can be used to assess 'vulnerable areas' to ischemic injury. In cardiac MRI adenosine stress test, perfusion ratio, more often referred to as 'myocardial perfusion reserve', is already utilized to evaluate the extent of ischemic injury in myocardial infarctions (16).

There are few studies evaluating foot perfusion using CT imaging, but these studies target patients with overt macrovascular dysfunction such as critical limb ischemia^{10,34}. The present study shows the feasibility of dynamic volume perfusion foot CT to detect early microvascular dysfunction in atherosclerosis model without macrovascular stenosis or occlusion, non-invasively and quantitatively. This is particularly important considering that currently available tools fail to provide information regarding anatomical area of decreased microperfusion, and monitor changes of microcirculation after treatment.

This study has several limitations. The rabbit model of atherosclerosis may have inherent differences in pathophysiology to human atherosclerosis as atherosclerosis is a multifactorial disease developed over a long time period with species-to-species variation. In addition, our rabbit model of atherosclerosis did not have significant macrovascular lesions in the large arteries which is found in human patients with peripheral artery diseases. However, the lack of macrovascular lesion allowed us to measure and compare TTP and perfusion parameters in the dermis, identifying possible early changes of atherosclerosis. Lastly, this study was also limited in only measuring one areas of foot perfusion due to the small size of the rabbit feet and a single dominant artery in the rabbit.

In conclusion, macrovascular reactivity and microvascular perfusion can be measured in a rabbit model of atherosclerosis by combination of perfusion foot CT using the upslope method and CRH. TTP in the tibial artery was prolonged during cuff-induced reactive hyperemic state in the atherosclerotic rabbits compared to healthy rabbits suggesting delayed vasoreactivity. Microvascular perfusion parameters such as perfusion value and perfusion ratio showed impairment in the atherosclerotic rabbits, suggesting ways

to detect at-risk tissues and early manifestation of atherosclerosis. Further human studies are warranted to clinically validate the usefulness of foot perfusion CT with CRH.

Abbreviations

CRH = Cuff-induced Reactive Hyperemia, PAE = Peak Arterial Enhancement, TTP = Time to Peak

Declarations

Author contributions

SH and JHL conceived of and designed the project. JHL conducted all wet lab work. CJY and WSC provided conceptual advice on analysis of data. HDM and JHL analyzed and interpreted the data. HDM, SH, JHL and SL prepared the manuscript, with contributions from all authors. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

(a) Study protocol for induction of atherosclerosis. (b) Timeline of CT perfusion protocol. (c) Color-coded perfusion map was reconstructed with perfusion CT. Microperfusion values were measured at the hyperperfusion tissue in the rabbit's heels (center). Region of interest is notated by blue circle on reconstructed image. (d) Tibial artery was measured in the foot, shown in red circle on non-reconstructed CT image.

Figure 2

Angiography of healthy rabbits (left) and cholesterol fed rabbits (right) showed no significant stenosis or occlusions in the aortoiliac and femorotibial arteries.

Figure 3

(a) Arterial time to peak. At baseline, cholesterol fed rabbits showed increased peak arterial enhancement (PAE) compared to healthy rabbits (354.6 ± 96.47 HU vs 255.51 ± 96.22 HU, $P < 0.001$). During CRH phase both groups showed an increased in PAE but intergroup differences were not significant. PAE = Arterial enhancement at CRH – Arterial enhancement at baseline. Housfield Unit (HU) (b) Arterial slope. Baseline arterial velocity was higher in cholesterol rabbits compared to normal rabbits (109.30 ± 25 vs 57.35 ± 33.44 HU, $P < 0.001$). During CRH phase, there was an increase in slope but no significant intergroup difference ($p = 0.977$). (c) Peak arterial enhancement. Cholesterol-fed rabbits showed no significant arterial time-to-peak differences at baseline compared to healthy rabbits (10.7 ± 1.82 HU vs 11.65 ± 2.32 HU, $P = 0.269$).

However, arterial TTP showed significant acceleration in healthy rabbits while no such reaction was observed in the cholesterol-fed group (p=0.025).

Figure 4

(a) Perfusion value showed significant increase in the healthy rabbits on CRH phase (35040.61 ± 17750.58 to 57582.43 ± 22585.11 HU; $P=0.008$) while no such surge was demonstrated in the cholesterol group (47427.59 ± 18968.08 to 46692.38 ± 19797.93 HU; $P = 0.878$). (b) Perfusion ratio, the ratio of perfusion at baseline vs. perfusion during CRH phase, showed significant drop in the cholesterol group compared to control group ($p=0.014$).

Figure 5

(a) Cholesterol-fed rabbits showed decreased endothelial cell density as measured by CD 31 expression. (b) Endothelial cell density as measured by CD 31 showed a lower density in the cholesterol-fed rabbits compared to control ($p<0.001$).