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Three-Dimensional Subharmonic Aided Pressure Estimation for Assessing Arterial Plaques in a Rabbit Model

Kibo Nam, PhD, Ji-Bin Liu, MD, John R. Eisenbrey, PhD, Maria Stanczak, MS, Priscilla Machado, MD, Jingzhi Li, MD

Department of Radiology, Thomas Jefferson University, Philadelphia, Pennsylvania

Zhaojun Li, MD,

Department of Vascular Ultrasound, Shanghai General Hospital, Shanghai, China

Ying Wei, MD,

Department of Ultrasound, Beijing Friendship Hospital, Beijing, China

Flemming Forsberg, PhD

Department of Radiology, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract

Objectives—To investigate 3-dimensional subharmonic aided pressure estimation (SHAPE) for measuring intraplaque pressure and the pressure gradient across the plaque cap as novel biomarkers for potentially predicting plaque vulnerability.

Methods—Twenty-seven rabbits received a high-cholesterol diet for 2 weeks before a balloon catheter injury to denude the endothelium of the aorta, followed by 8 to 10 weeks of the high-cholesterol diet to create arteriosclerotic plaques. SHAPE imagings of the resulting plaques were performed 12, 16, and 20 weeks after injury using a LOGIQ 9 scanner with a 4D10L probe (GE Healthcare, Milwaukee, WI) before and during an infusion of Definity (Lantheus Medical Imaging, North Billerica, MA) and Sonazoid (GE Healthcare, Oslo, Norway). The ratios of the maximum subharmonic magnitudes at baseline and during the infusion were correlated with the intraplaque pressure and pressure gradient across the plaque cap obtained from direct measurements.

Results—Ten rabbits died prematurely after the balloon injury procedure or due to toxicity from the high-cholesterol diet, whereas 2 rabbits were excluded for other conditions. Five rabbits were scanned in the 12-, 16-, and 20-week groups, respectively. Even after 20 weeks, the plaques that developed were very small (mean $\pm$ SD, $0.9 \pm 0.4 \times 0.14 \pm 0.05$ cm). Definity performed better than Sonazoid in this application but still only achieved a moderate correlation with pressure across the plaque cap (Definity, $r = -0.40$; Sonazoid, $r = 0.22$) and intraplaque pressure (Definity, $r = -0.19$; Sonazoid, $r = -0.11$).

Conclusions—Initial findings from plaque pressure estimation using 3-dimensional SHAPE technique showed only moderate correlations with reference standards, but that may have been due to weaknesses in the animal model studied.

Keywords

arterial plaque; contrast-enhanced ultrasound; pressure estimation; subharmonic imaging; vulnerable plaque

Atherosclerosis is a systemic arterial disease and a leading cause of vascular disease worldwide.¹ Serious and potentially lethal vascular diseases, such as acute coronary syndrome and ischemic stroke, are usually caused by atherothrombosis, which is acute thrombosis superimposed on a chronic atherosclerotic plaque with a disrupted or eroded surface.² Thus, early detection of rupture-prone or so-called vulnerable plaques would advance vascular disease prevention.³

To predict vulnerability, attempts have been made to assess the risk factors associated with atherothrombotic plaques. Atherothrombotic plaques are mainly composed of a connective tissue extracellular matrix, including collagen, proteoglycans, and fibronectin elastic fibers.^{4–6} Each plaque shows a heterogeneous distribution of these components, which mostly affect the intima, but secondary changes occur in the media and adventitia as well, including growth of vasa vasorum.^{5,7–9} Burke et al¹⁰ examined the hearts of 113 men with coronary disease who had died suddenly. They found that among 59 men who had an acute coronary thrombus, 41 resulted from rupture of a vulnerable plaque, and 18 resulted from the erosion of a fibrous plaque.¹⁰ The ruptured plaques had a lipid-rich core under the thin fibrous cap (95% of ruptured caps were < 64 μm), whereas fibrous plaque had mainly smooth muscle cells and proteoglycans.¹⁰ Hence, a vulnerable plaque was defined as a fibrous cap less than 65 μm thick with an infiltrate of macrophages with or without plaque rupture.¹⁰ Also, the cap of a vulnerable plaque is often thinnest at the shoulder region, where macrophages and mast cells accumulate.⁶ The core of a vulnerable plaque is known to be soft, which may be more vulnerable to rupture, as it may not be able to resist the imposed circumferential stress.⁶ Other characteristics include active inflammation, endothelial denudation with superficial platelet aggregation, a fissured plaque, and the presence of high-grade coronary stenosis, as well as a superficial calcified nodule, the presence of plaque hemorrhage, endothelial dysfunction, and positive remodeling of the artery.^{3,6,11}

Other studies investigated the local hemodynamic conditions to characterize a vulnerable plaque. As major local hemodynamic factors, endothelial shear stress and circumferential tensile stress were suggested.^{12–14} Endothelial shear stress is induced by the bloodstream movement directed to the nonmoving vessel wall, causing strain parallel to the surface of endothelial cells.¹³ A heterogeneous local endothelial shear stress is applied to the stenotic plaques lengthwise so that the upstream shoulders, the neck, and the downstream shoulders of plaques receive low, high, and low/oscillatory endothelial shear stress, respectively.^{14–16} Cicha et al¹⁷ observed that 86% of ruptured plaques occurred upstream in a study of 80 human carotid specimens.

Plaque neovascularity and the subsequent pressure increase it induces have also been proposed as a measure of plaque vulnerability. In the study by Cicha et al,¹⁷ neovascularization was found in 58 plaques, and 43 of them were observed at the upstream side of the lesions. Similarly, 31 of 51 plaques with intraplaque hemorrhage showed the hemorrhage only at the upstream side of the plaque, whereas 20 showed it at both sides.¹⁷ A circumferential stress is caused when the blood pressure is applied perpendicular to the vessel wall and stress in the range of 300 to 500 kPa results in plaque rupture.¹³ The persistent and repetitive cyclic stress may weaken a plaque cap, and combined with factors of plaque composition and cap thickness, it can lead a plaque to sudden rupture.²

The studies attempting to predict vulnerable plaques have evolved from focusing on morphologic determinants of instability to including extrinsic stressors.¹⁸ However, Stefanadis et al¹⁸ found that most of the current studies still focused on the plaque structure using imaging modalities, and none of them established criteria for assessing plaque vulnerability in the broader sense. Hence, parameters that can reflect the intrinsic and extrinsic conditions together are essential to detect plaque vulnerability more accurately.

According to the previous findings, a plaque ruptures when the pressure and stress within the plaque exceed the strength of the fibrous cap.^{13,17,18} Also, neovascularity and hemorrhage within a plaque increase the pressure within the plaque.^{17,19–21} Thus, intraplaque pressure may reflect the structural condition of a plaque, whereas the pressure gradient between the plaque and the blood vessel may reflect the local hemodynamic conditions affecting the plaque. The overall hypothesis of this study was that vulnerable plaques would have higher intraplaque pressure as well as a higher pressure gradient across the plaque cap compared to normal plaques.

Contrast-enhanced ultrasound (US) is widely used for evaluations of blood perfusion within tumors, the liver, kidneys, and other organs.^{22–24} It has also been used to estimate the ambient pressure using microbubbles as pressure sensors. Microbubbles oscillate, producing multiple harmonics of the fundamental frequency, and these oscillations change depending on the ambient pressure changes. It was previously shown that there is an inverse relationship between the microbubble signal amplitude at the subharmonic frequency and ambient pressure.^{25–27} The sensitivity of this inverse relationship varies with the applied acoustic pressure. Thus, the ambient pressure is estimated after the transmit acoustic settings are adjusted to maximize the sensitivity of the inverse relationship.^{25–27} This technique is called subharmonic aided pressure estimation (SHAPE) and has been successfully applied for assessing portal hypertension, monitoring neoadjuvant chemotherapy responses, and estimating intracardiac pressure.^{26–28} In this study, the SHAPE technique was used to estimate the intraplaque pressure and pressure gradient across the plaque cap in a rabbit model.

Materials and Methods

This study was compliant with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee. The study protocol was supervised by our Laboratory Animal Services Department.

Animal Model

At the beginning of the study, 9 Watanabe heritable hyperlipidemic (WHHL) rabbits were studied. The WHHL rabbit shows hypercholesterolemia, due to the lack of low-density lipoprotein receptors, and has a very similar lipoprotein metabolism as humans.²⁹ This animal model has been widely used in plaque-related studies.^{30–32} However, a very limited number of vendors were able to provide WHHL rabbits at the time of the study, and the supply was not sufficient for the entire study. Thus, New Zealand white (NZW) rabbits were chosen for the rest of the study, and 18 of them were studied. The NZW rabbit is also a popular model for human atherosclerosis studies.^{33–35} It develops hypercholesterolemia within a few days of dietary supplementation.³³

For the formation of a plaque, rabbits received a high-cholesterol diet (1–2%) for 2 weeks before a balloon catheter injury to denude the endothelium of the aorta, followed by 8 to 10 weeks of the high-cholesterol diet to create arteriosclerotic plaques.³⁴ The rabbits were premedicated with 3 to 5 mg/kg xylazine hydrochloride (Gemini; Rugby Laboratory, Rockville Centre, NY) and 30 to 40 mg/kg ketamine hydrochloride (Ketaset; Aveco, Fort Dodge, IA) or 0.25 to 1.0 mg/kg acepromazine (PromAce; Boehringer Ingelheim Vetmedica, Inc, St Joseph, MO), and general anesthesia was applied with isoflurane (Iso-thesia; Abbott Laboratories, Chicago, IL) for a balloon de-endothelization (4%–5% for induction and 0.5%–2% during the procedure). The balloon injury was performed at the aorta about 2 cm below the origin of the renal arteries. After a balloon injury, the rabbits were divided into 3 groups: 12 weeks, 16 weeks, and 20 weeks after injury.

Contrast Agents

Two US contrast agents were used in this study: Definity (Lantheus Medical Imaging, North Billerica, MA) and Sonazoid (GE Healthcare, Oslo, Norway). These contrast agents were selected on the basis of previous in vitro work, which has shown relatively high sensitivity of the subharmonic signal change to the ambient pressure change compared to other agents.³⁶ Definity is composed of lipid-coated microspheres filled with octafluoropropane gas. Its mean diameter range is between 1.1 and 3.3 μm , and 98% are less than 10 μm .³⁷ Sonazoid is also composed of lipid-coated microspheres but filled with perfluorobutane gas. Its diameter typically ranges between 1 and 5 μm , and 99.9% have a diameter of less than 7 μm .³⁸ These US contrast agents were chosen on the basis of previous studies involving SHAPE.^{39,40} Definity was infused intravenously with 1.3 mL of Definity/50 mL of saline at infusion rates of 4 to 10 mL/min (titrated to effect). Sonazoid was mixed with 2 mL of water for injection and coinjected with saline via a split intravenous line at an infusion rate of 0.09 mL/kg/h.

Invasive Pressure Measurement Systems

As a reference standard, the aortic and intraplaque pressures were directly measured with a 0.75F fiber-optic pressure catheter (FISO Technologies, Quebec City, Quebec, Canada) and an intracompartmental pressure-monitoring system (Stryker, Newbury, Berkshire, England), respectively. The pressure gradient across the plaque cap was obtained by subtracting the aortic pressure from the intraplaque pressure.

Ultrasound Examinations

The rabbits were anesthetized with isoflurane before the experiment, and anesthesia was maintained by 2–4% of isoflurane delivered through an endotracheal tube (titrated to effect). The body temperature was maintained with a warming pad during the study. The optic pressure catheter was inserted into the aorta through a femoral artery, and the sensor tip was located near the plaque guided by US. The aortic pressure was measured for 5 seconds before the administration of the contrast agent and digitized by the manufacturer's software installed on a computer connected to the pressure system.

After baseline imaging, the rabbits received a continuous infusion of the first contrast agent (Definity or Sonazoid, randomly chosen) through an ear vein. Contrast-enhanced US examinations were performed with a modified LOGIQ 9 scanner with a 4D10L probe (GE Healthcare, Milwaukee, WI). Modified software allowed collection of radiofrequency (RF) data from a 3-dimensional (3D) subharmonic mode (transmitting at 5.8 MHz and receiving at 2.9 MHz using a pulse inversion technique).²² The acoustic output setting was optimized for the corresponding contrast agent at the location of the plaque for SHAPE using built-in optimization software on the scanner based on the work by Dave et al.⁴¹ The inverse relationship between ambient pressures and the subharmonic signal magnitude is affected by the transmitted acoustic pressure, and an individual optimization of the acoustic setting for SHAPE is necessary.²⁵ After that, color Doppler US was applied to rupture the microbubbles using its high duty cycle, longer pulses, and high mechanical index settings.⁴² Once microbubble clearance was confirmed visually, RF data for 3D SHAPE were collected without microbubbles. Then rabbits received the microbubble infusion for 3D SHAPE RF data collection. The RF data were collected from an area including the plaque. During the infusion, pressure in the aorta was measured for 5 seconds. The aortic pressure was obtained by averaging systolic and diastolic pressures measured for that period. This whole process was repeated for the second US contrast agent (Figure 1). Finally, the intraplaque pressure was measured in 5 to 8 locations within the plaque using a side-ported and noncoring 18-gauge needle attached to the Stryker measurement system. The final intraplaque pressure was obtained by averaging those measurements.

Data Analysis for Pressure Estimation Using 3D SHAPE

The collected 3D SHAPE RF data were analyzed offline with MATLAB version R2015b software (The MathWorks, Natick, MA). The RF data consisted of multiple 3D volumes (5–25 volumes) over time, and each 3D volume was composed of multiple 2-dimensional elevational images (7–15 slices depending on the volume size). Regions of interest were selected around the plaque (or thickened wall area) in each elevational plane. A fast Fourier transform (FFT) was applied to each RF A-line within each region of interest. The resultant FFT values were averaged laterally and elevationally. This process was repeated for all available volumes over time. Finally, a single FFT result was obtained by averaging across volumes. Using the final FFT result, the maximum subharmonic frequency magnitude was calculated before and during the infusion. Finally, the ratio of the subharmonic signal during infusion to the pre-infusion signal was determined. The obtained 3D SHAPE result from a given plaque area were compared to the intraplaque pressure by linear regression analysis in MATLAB. Also, the gradient of the 3D SHAPE results, which was calculated by subtracting

the 3D SHAPE result of the aorta from that of the plaque area, was compared to the pressure gradient across the plaque cap. The correlation coefficient between the gradient of 3D SHAPE results and pressure gradient across the plaque cap was calculated.

Results

A number of adverse events were encountered involving 10 rabbits (4 WHHL and 6 NZW rabbits) of the 27 studied in this project. Six rabbits died within 24 hours after the balloon injury procedures, and another 4 died 8 to 12 weeks into the postinjury period, due to liver failure caused by toxicity from the high-cholesterol diet. The total cholesterol levels in the 2 sampled rabbits were 1321 and 2117 mg/dL, respectively. Two additional NZW rabbits were excluded from the study because of preexisting conditions. Thus, 5 rabbits were scanned in each of the 12-, 16-, and 20-week groups. The weight range of these rabbits was 2.9 to 4.4 kg with a mean value of 3.4 kg.

Even after 20 weeks, the size of the plaque was very small (mean $\pm$ SD, $0.9 \pm 0.4 \times 0.14 \pm 0.05$ cm) and mainly consisted of wall nodularity or thickened walls rather than well-defined plaques. Example images from a rabbit in the 20-week postinjury group are shown in Figure 2. The locations of the plaques or thickened wall areas in subharmonic images were confirmed initially by using conventional B-mode imaging of the aorta starting about 2 cm below the renal arteries, which was then used to guide the tip of the optic pressure sensor to that location. The plaque or thickened wall areas were mostly hypoechoic when imaged in the subharmonic mode after the infusion of contrast agents (Figure 2). The aortic pressure was averaged over 3 to 5 seconds and ranged between 20 and 62 mmHg with a mean value of 38 mmHg for Definity and between 20 and 60 mmHg with a mean value of 36 mmHg for Sonazoid. Without any contrast agent, pressures ranged from 27 to 87 mmHg. The intraplaque pressure was between 5 and 32 mmHg, and the mean value was 13 mmHg.

The 3D SHAPE results showed heterogeneity over the 2-dimensional elevational planes, as shown in Figure 3. Definity performed better than Sonazoid in this application but still only achieved a moderate correlation with pressure across the plaque cap (Definity, $r = -0.40$; $P = 0.14$; Sonazoid, $r = 0.22$; $P = 0.42$) and intraplaque pressure (Definity, $r = -0.19$; $P = 0.50$; Sonazoid, $r = -0.11$; $P = 0.70$). Figure 4 shows the correlations between the gradient of 3D SHAPE results and pressure gradient across the plaque cap during the infusion of Definity and Sonazoid.

Discussion

A number of unexpected events occurred in both rabbit species. The high-cholesterol diet caused cardiomyopathy, coronary artery disease, aortic atherosclerosis, valvular disease, pulmonary atherosclerosis, and hepatic steatosis. Some animals refused to eat the high-cholesterol diet and had weight loss. Thus, the initial diet with 2% cholesterol was changed to 1% cholesterol. The lower-cholesterol diet reduced the number of adverse events, but they still occurred because of the long plaque formation period. Even in the longest plaque formation period (20 weeks after injury), plaques were small and not always guaranteed to form.

Due to the small size of the plaques, in some cases the subharmonic signal did not change much with the acoustic output. Hence, the chosen acoustic output settings may not have been optimal for the SHAPE technique. Also, there were challenges in correlating the 3D SHAPE results with direct measurements of intraplaque pressure and pressure gradients across the plaque cap. The intraplaque pressures were measured in various locations within a plaque using a fine needle, but their locations could not be registered with those of the SHAPE results. The plaques were generally very small (in the beam direction), which necessitated including some of the surrounding area around the plaques (Figure 3) to calculate accurate FFTs and, therefore, SHAPE results. The aortic pressure was also obtained by averaging systolic and diastolic pressures because these measurements were not synchronized with 3D SHAPE data collection. These issues could be the reason that only a moderate or poor correlation was achieved and even an unexpected (given our prior experiences)^{22,25,26,28,39–41} positive correlation in the case of the SHAPE gradient from Sonazoid.

Although the correlation was moderate, the gradient of 3D SHAPE results obtained with Definity did show an inverse relationship with the pressure gradients across the plaque cap (Figure 4). This may imply that the local hemodynamic conditions around a plaque can be reflected in the SHAPE results. The SHAPE results from Definity showed better correlations with pressure measurements than those from Sonazoid even with their similar bubble size distributions, which may have been due to differences in microbubble characteristics such as the resonance frequency, attenuation, and scattering.⁴³

In most of the cases studied, plaques were hypoechoic, even with enhancement from a contrast agent. This could have been because the contrast agents could not enter due to the small size of blood vessels within plaques, although other researchers have shown contrast-enhanced US enhancement of the vasa vasorum (albeit not in these animal models).⁴⁴ Alternatively, these plaques were more calcified wall abnormalities than regular plaques and may have had underdeveloped neovascularity. The current SHAPE software uses 2.6 MHz as a subharmonic frequency, and it did not provide high-enough resolution to assess the small plaques.

In conclusion, this study was conducted to provide a proof of concept of 3D SHAPE for plaque pressure estimation. The biggest obstacle was the formation of plaques in our animal model. Initial findings from 3D SHAPE plaque pressure estimation show only moderate correlations with reference standards, but that may have been due to weaknesses in the animal model studied.

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Abbreviations

FFT fast Fourier transform

NZW	New Zealand white
RF	radiofrequency
SHAPE	subharmonic aided pressure estimation
3D	3-dimensional
US	ultrasound
WHHL	Watanabe heritable hyperlipidemic

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Figure 1.
Study procedure and timeline.

Figure 2.

Example images of the plaque area from a rabbit in the 20-week group. The boxes indicate a plaque area in the B-mode image (**A**; the bar on the right indicates 0.5 cm), subharmonic image without a contrast agent (**B**), subharmonic image with Definity (**C**), and subharmonic image with Sonazoid (**D**). The dashed arrows in **B–D** indicate the walls of aorta. The aorta is dark in **B**, whereas it is filled with microbubble echoes (white dots) in **C** and **D**. The aorta is revealed to be thickened and whitened in the photograph (**E**). The size of plaque was 1.25×0.14 cm.

Figure 3.

The normalized (for display purposes) magnitude of the maximum subharmonic signal during infusion was overlaid on the 2-dimensional subharmonic images using the color bar (showing values of 0–1 in arbitrary units) on the right. The location of the plaque was confirmed by conventional B-mode imaging. In this example, the midplane of the 3D volume is shown in **A** and **B**, whereas **C** and **D** show the next elevational plane of it. The solid and dashed boxes indicate the plaque and aorta, respectively.

Figure 4.

Correlations between the gradient of 3D SHAPE results and pressure across the plaque cap during the infusion of Definity (A) and Sonazoid (B). The gradient of 3D SHAPE results was calculated by subtracting the maximum subharmonic magnitude ratio (during infusion to before infusion) of the aortic area from that of the plaque area.