Atherosclerosis: Open Access

Miura, Atheroscler open access 2021, 6:2

Review Article Open Acces

Histological and Mechanical Information Based on Biochemical Alterations of Cardiovascular Diseases Using Scanning Acoustic Microscopy with Proteinases: A Novel Technique for Cardiovascular Research

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Abstract

Scanning Acoustic Microscopy (SAM) simultaneously provides structural and mechanical information from a histological glass slide. It supplies objective evidence of damage or degeneration because the Speed-of-Sound (SOS) of each portion correlates with its stiffness. Moreover, comparing SOS values before and after proteinase treatment discloses the susceptibility of the breakdown. We used this method in a cardiovascular study on atherosclerosis, aging, myocardial infarction, cardiac valve dysfunction, and mechanical rupture. With aging, smooth muscles and elastic fibers of arteries and the aorta decrease in number, resulting in reduced SOS values. Although atherosclerosis makes tissues stiffer, mainly in the intima, the stiffness of the outside medial layer quantitatively decreases comparable to SOS values. Fibrosis after inflammation or infarction usually becomes stiffer than the original state. Older fibrosis shows more resistance to proteinase digestion, which indicates the additional modification in the proteins of the old fibrosis. Mechanical stress causes tissue fracture, such as rupture of the tendinous cord of the mitral valve. The ruptured portion shows an irregular array of fibers with loss of cells and myxoid degeneration where reduced SOS values and accumulation of some extracellular matrix components appear. Comparing Aortic Stenosis (AS) with regurgitation (AR) of the aortic valve, the fibrosa of both valves was stiffer than that of the normal valves. The fibrosa of AR is thinner but stiffer and more resistant to proteinase digestion than that of AS. Advanced glycation end-products are deposited more in the AR valve. Histological observation using SAM can reveal mechanical alterations based on biochemical abnormality.

Keywords: Scanning acoustic microscopy; Speed of sound; Atherosclerosis; Aging; Myocardial infarction; Cardiac valve; Proteinase

Introduction

Scanning Acoustic Microscopy (SAM) is a histological imaging modality using high-frequency (>80- MHz) ultrasound [1,2]. The Speed-of-Sound (SOS) through tissues differs in constituents, making tissue imaging possible by plotting the SOS values on the screen. Since SOS values correspond to tissue stiffness, SOS imaging shows the structure and mechanical properties of tissues. The cardiovascular system functions on structural and mechanical properties of the heart and blood vessels. Quarte and Lemons at Stanford University (CA, USA) have developed a SAM, the basic design of which is now used in the biomedical field [3,4]. To date, SAM using more than an 80-MHz ultrasound has been used in vascular and heart pathological examinations.

The superiority of SAM to Light Microscopy (LM) is shown in Table 1. First, no staining is necessary to obtain images. Only unstained flat sections in approximately 10-μm thickness are scanned through water as a coupling medium to measure SOS. Both fresh-frozen and fixed sections can be scanned. Moreover, repeated observations are possible by using the same slide. In addition, cultured cells on dishes could be observed using the impedance mode [1,5]. Second, the mechanical properties of the stiffness of each component on histology are visible in color images. SOS, attenuation of sound, and tissue thickness are calculated and plotted to quickly make images on the screen. Third, statistically evaluating images is easy by comparing the values of each area. Each image is made of numerical data; therefore, mathematical analysis is precise and easy. Fourth, conformational changes after treatment, such as enzymatic digestion and tissue staining [6], can be followed using the same slide. Comparing SOS values before and after treatment assesses the treatment's effects [7,8]. SOS comparison after proteinase digestion exhibits the vulnerability of tissues to breaking damage, and acoustic staining exposes the biochemical components of each image.

We used this cardiovascular research method on atherosclerosis, aging, myocardial infarction, cardiac valve dysfunction, and mechanical rupture. This mini-review article will present the recent study to elucidate various cardiovascular diseases using the SAM imaging modality.

The relationship between SOS and stiffness

The SOS is the distance traveled per unit of time by a sound wave as it propagates through materials. Sound waves in solids are composed of compression and shear waves. The SOS of compression waves in solids is determined by the medium's compressibility, shear modulus, and density (Wikipedia. Speed of sound, https://en.wikipedia.org/wiki/Speed_of_sound, 2020 (accessed Jan. 15, 2021). In solids, compression waves are analogous to those in fluids, depending on the compressibility and density, but with the additional factor of shear modulus, which affects compression waves due to off- axis

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Received February 04, 2021; **Accepted** February 17, 2021; **Published** February 24, 2021

Citation: Miura K (2021) Histological and Mechanical Information Based on Biochemical Alterations of Cardiovascular Diseases Using Scanning Acoustic Microscopy with Proteinases: A Novel Technique for Cardiovascular Research. Atherosclerosis: An Overview. Atheroscler Open Access. 6:149.

	LM (Light Microscopy)	SAM (Scanning Acoustic Microscopy)
Medium	Light	Ultrasound
Image	Analogue	Digital
Staining	Necessary	Not necessary
Special stain for specific materials	Possible	Possible
Immunostaining	Possible	Possible
Protease treatment	Not detected	Detected
Mechanical property	Not detected	Detected (SOS, AOS)
Observation of time lapse	Possible	Difficult
Surface state of section	Tolerance	Smooth surface necessary
Tissue thickness	Wide range,1 μm~15 μm	Limitted range, ~10 μm
In vivo observation of culture cells	Possible	Possible by Impidance mode
Thickness measurement	Impossible	Possible
Observation area	Free and adjustable	Limitted
Imaging with computer	Not necessary	Necessary
Time to obtain images	Simultaneous	At least a few min
Standard areas for observation	Not necessary	Absolutely necessary

Table 1: Typical features of Scanning Acoustic Microscopy (SAM) and Light Microscopy (LM).

energies, which can adequately influence the tension and relaxation in compression. In our SAM system, we mainly use compression waves to measure the SOS through tissues.

The Newton–Laplace equation gives the SOS:

$$c = \sqrt{\frac{K}{\rho}}$$

Where c indicates SOS, K indicates elastic bulk modulus or the coefficient of stiffness, and ρ indicates density.

The SOS increases with the stiffness (the resistance of an elastic body to deformation by an applied force) of the material and decreases with an increase in density. In human soft tissues, density is almost

1 g/cm^3 (IT'IS foundation, Database of tissue properties, density, https://itis.swiss/virtual-population/tissue-properties/database/density/, 2020 (accessed Jan. 15, 2021)) comparable to that of water. Saijo has reported that the density of the coronary artery ranges from 1.078 to 1.086 g/cm^3 [9]. Therefore, the SOS through soft tissues correlates positively with their stiffness (IT'IS foundation, Database of tissue properties, Speed of sound. https://itis.swiss/virtual-population/tissue-properties/database/acoustic-properties/speed-of-sound/, 2020 (accessed Jan. 15, 2021).

The principle of SOS measurement using SAM

Figure 1 shows the principle of a scanning acoustic microscope. Samples are placed on glass slides, and distilled water is used for coupling fluid between the transducer and specimen [10]. The transducer is used to transmit and receive a single-pulsed wave signal and scans X–Y directions on the slide. Waveforms reflected from the sample's surface and bottom are compared to measure the SOS and thickness of each point [11,12]. The waveform from a glass surface without the sample serves as the reference, with the SOS only through water (1495 m/s is used as a standard value).

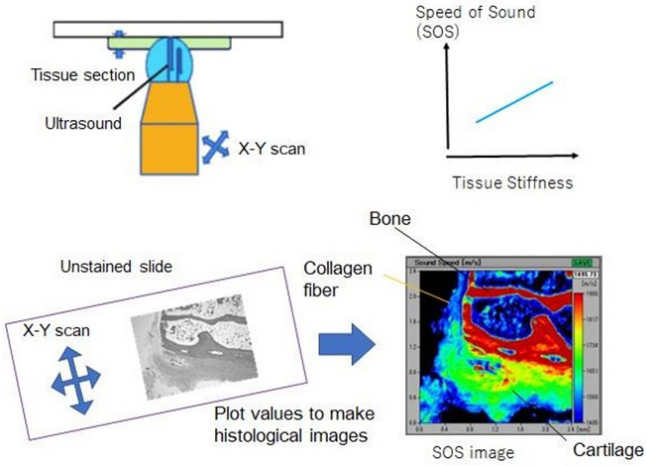


Figure 1: Principle of a scanning acoustic microscope: Samples are placed on glass slides, and distilled water is used as a coupling fluid between the transducer and specimen. The transducer is used to transmit and receive a single-pulsed wave signal and scans X–Y directions on the slide. Waveforms reflected from the surface and bottom of the sample are compared to measure the Speed-of-Sound (SOS) and thickness of each point. The waveform from a glass surface without the sample serves as the reference, with the SOS only through water; 1495 m/s is used as a standard value.

Both Frozen and Formalin-Fixed, Paraffin-Embedded (FFPE) sections can be observed. Flat-surface 10- μm-thick sections are suitable for observation to avoid irregular reflections and obtain precise SOS measurements. The mechanical scanner is arranged so that the ultrasonic beam is transmitted over the specimen to provide SOS values from each point. The mean SOS values of each point are plotted on the screen to make the SOS image in color. A histological map of stiffness shows that stiffer areas are red and softer areas are

blue in color according to color coding on the screen. Although FFPE specimens show higher SOS values than fresh ones, SOS values are stable irrespective of the length of formalin fixation (from 1 day to 3 months) [13].

The application of SAM in cardiovascular research

To date, we have used SAM in cardiovascular studies on the aortae, muscular arteries, cardiac valves, and heart. Figure 2A is a representative SOS image of the normal aortic valve and thoracic aorta. Blood vessels and cardiac valves comprise a layered structure, where functional abnormalities accompany the disarray of structures. SOS images correlate well with LM images, and a high-value structure agrees with collagen and smooth muscle fibers. Atherosclerosis of the aorta and muscular arteries in which lipids accumulate with fibrosis and calcification with aging causes structural and functional changes with wall stiffness and hypertension. Ischemic changes or deposition diseases in the heart show localized alterations of both conformational and functional disorders. Cardiac valves lead to stenosis, regurgitation, and rupture. SAM, which simultaneously detects structure and functional abnormalities, is a suitable tool to analyze and compare the lesions for these cardiovascular disorders.

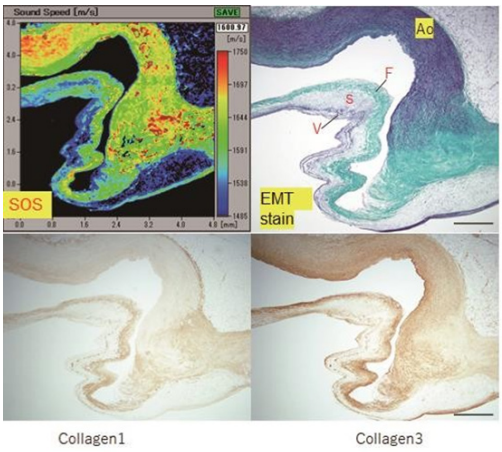


Figure 2A: Scanning acoustic microscopy (SAM) images of the aortic valve (AV).
The AV of a 21-year-old male in SAM and light microscopy (LM) (Masson's trichrome stain) images and the corresponding immunostaining with anti-collagen type 1 and type 3 antibody.
The AV consists of three-layered structures, including the fibrosa (F), spongiosa (S), and ventricularis (V), where SAM images correspond well to LM images. Collagen and elastic fibers, green and black in LM images, exhibit high- and low-SOS images. The nearby aorta (Ao) comprises regular parallel elastic fibers and smooth muscles. The thick collagen fibers at the base exhibit greater SOS values. The higher valued portions of SOS are rich in type 1 or type 3 collagen. The scale bars represent 1 mm.

Atherosclerosis observed by SAM

Figure 2B shows the comparative images of young and old thoracic aortae in SOS and LM images. The old aorta with severe atherosclerosis shows the irregular distribution of high- and low-SOS areas, which correspond to the collagen-rich fibrous and lipid-rich transparent portions, respectively. However, the young aorta displayed stable three-layered structures, such as the intima, media, and adventitia. The medial layer consisting of thick smooth muscles appears to have a great homogenous value of SOS.

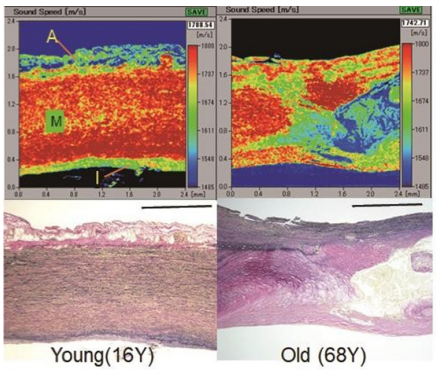


Figure 2B: SAM (upper) and LM (lower) images of young and old thoracic aortae. The young aorta consists of three regular layers, including the intima (I), media (M), and adventitia (A). The old aorta is composed of irregular layers with severe atherosclerotic plaques. Red-colored portions in LM images of Verhoeff–Van Gieson elastic staining (EVG) indicate collagen fibers, and black-colored ones correspond to elastic fibers. The scale bars represent 1 mm.

SOS alterations correlate with structural and functional changes.

SOS scores correlate negatively with aging and medial degeneration grade scores [7]. Dissected aortae showed higher SOS values and medial degeneration grade scores without age correlation. Older and dissected aortae express specific Extracellular Matrix (ECM) components to compensate for mechanical weakness [7].

Muscular arteries, such as the Renal Artery (RA) [14] (Figure 2C) and coronary artery [9], with atheromatous plaque showed similar aortic images. SOS alterations correlated with structural and functional changes. The SOS values of the tunica media and internal and external elastic laminae show a reverse correlation with age and blood pressure, indicating mechanical weakness of the tunica media with aging. The RA displays remodeling, such as external expansion of the media with inward hypertrophy of the intima. As a consequence, the outer structure of the RA changes from round to oval with medial dilatation.

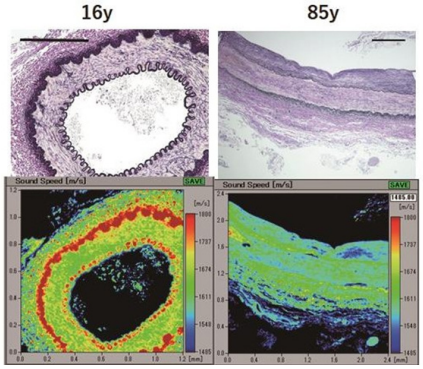


Figure 2C: An LM image of the RAs (left:16-year-old, right:85-year-old) in EVG and their corresponding SAM images.
The young RA comprises well-organized layered structures, such as the intima, media, and adventitia, divided by the internal and external elastic laminae, which are clearly shown in the SAM and LM images. Both notched elastic laminae offer the most outstanding value. The media exhibits a fine filamentous texture, and the adventitia displays a rough folded texture. The old RA is elongated and consists of a three-layered structure with thick intima and smooth internal and external elastic laminae. The SOS values of all components are lower than those of the young RA. The scale bars represent 0.5 mm.

Susceptibility to proteinases digestion

A state of tissue remodeling, such as inflammation, necrosis,

and regeneration, must remove old constituents. Susceptibility to breakdown is an essential issue for tissue metabolism. SAM is a satisfactory method to evaluate this property.

Figure 3A shows the comparative results after pepsin digestion between young and old RA. A young RA exhibits a rapid reduction in SOS after digestion, whereas an old RA displays no remarkable changes. An old RA is more resistant to proteinase digestion than a young RA. Regarding aortic valves (Figure 3B), old aortic valves are more resistant to proteinase digestion than young ones. Old tissues must have a different modification of proteins to protect digestions. More advanced glycation end-products were found in old aortic valves with slow collagen turnover [15].

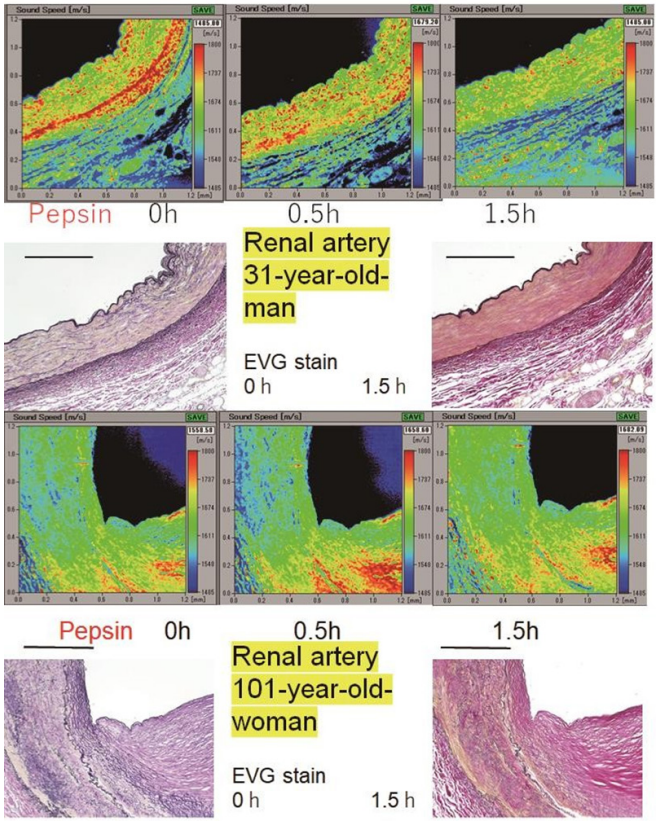


Figure 3A: Scanning acoustic microscopy (SAM) images after pepsin digestion. Speed-of-sound (SOS) alteration after pepsin digestion of young and old renal artery (RA)
The young RA with little atherosclerosis shows a remarkable reduction in SOS value after digestion whereas the old RA with atherosclerosis displays stable SOS after digestion. Fibrous areas are resistant to pepsin digestion. The corresponding light microscopic (LM) images on the lower row are Verhoeff– Van Gieson elastic stained (EVG). The scale bars represent 0.5 mm.

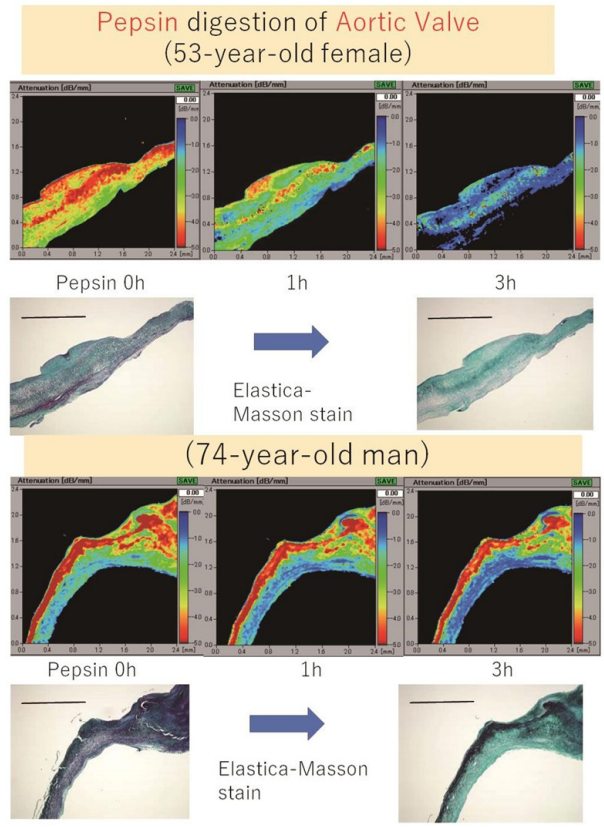


Figure 3B: The cusp of the aortic valve of a normal individual and that with aortic regurgitation in SAM images with pepsin digestion.
The normal aortic valve of a 53-year-old female comprises a three-layered structure, displaying a gradual reduction in SOS values after pepsin digestion. However, the aortic valve with regurgitation of a 74-year-old male reveals resistance after digestion. The scale bars represent 1 mm.

Mechanical rupture of the aorta and mitral valve

Mechanical rupture, such as dissection of the aorta and rupture of a tendinous cord of the mitral valve (Figure 4), was investigated using SAM. Dissected aortas showed higher SOS values without age correlation and more susceptibility to collagenase digestion than normal aortas [7]. Specific types of ECM proteins are expressed differently among individuals with Marfan syndrome and non-Marfan syndrome cases to compensate for mechanical weakness [7]. A ruptured tendinous cord displays an irregular array of fibers with loss of cells with myxoid changes. Myxoid portions correspond to the low-SOS areas where ECM components, such as vitronectin and fibronectin, accumulated [15].

Old and acute necrosis of cardiac muscles

SOS images are available for estimating myocardial infarction. Figure 5 compares SOS and LM images of old and acute myocardial infarction. Healed areas with fibrous scar (old infarction) correspond to high-SOS portions, where values are stable after pepsin digestion, whereas normal myocardial areas reduced their SOS values after digestion. Measurements of acute myocardial infarction with surrounding edematous parts show a rapid reduction in SOS values after pepsin digestion.

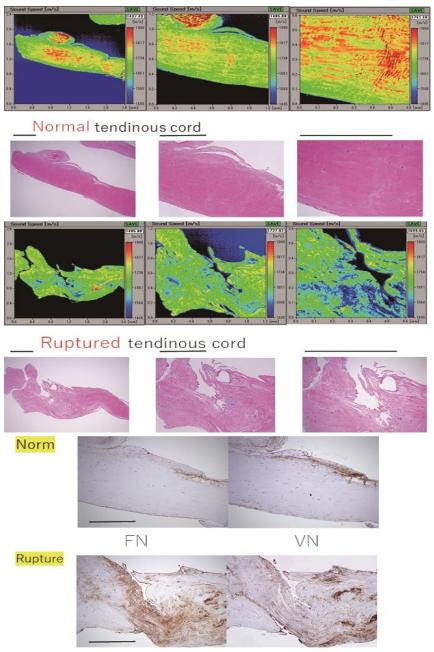


Figure 4: Rupture of a tendinous cord of the mitral valve compared with a normal control.
A ruptured portion with a crack shows an irregular array of fibers with loss of cells with myxoid changes. Myxoid parts correspond to the low-speed-of-sound (SOS) areas where extracellular matrix components, vitronectin and fibronectin, accumulated. The normal tendinous cord consists of a regular collagen fiber array which displays high-SOS values with no deposition at the extracellular matrix. The scale bars represent 0.5 mm.

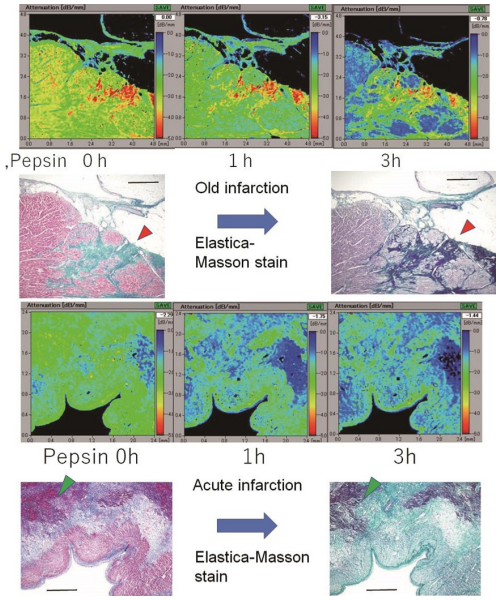
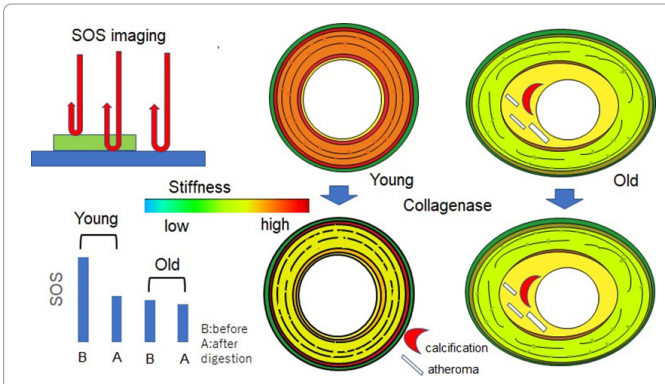


Figure 5: Alterations in old and acute myocardial infarction after pepsin digestion in scanning acoustic microscopy and light microscopy (LM) (Masson's trichrome stain) images.
Healed areas with a fibrous scar (arrowheads) correspond to high-speed-of-sound (SOS) portions where values are stable after pepsin digestion, whereas normal myocardial areas had reduced SOS values after digestion. Measurements of acute myocardial infarction with surrounding edematous parts show a rapid reduction in SOS values after pepsin digestion. Upper, old infarction; lower, acute infarction. The scale bars represent 1 mm.



Graphic abstract: Summary of the method of SOS imaging with collagenase
Speed of sound (SOS) through tissues differs among constituents because stiffer or elastic tissues show higher SOS. Muscular arteries such as renal arteries lose elasticity with aging, in which the medial layer of the elderly diminishes smooth muscles and display lower SOS than that of the young. After collagenase digestion, SOS values of the media of the young decrease more significantly than those of the elderly. Therefore, the medial layer of the young is more susceptible to collagenase. Stiffness and susceptibility to collagenase are statistically comparable between lesions.

Limitations of this observation method

This method of SAM with proteinases has several limitations that should be acknowledged. First, the fixation effects may influence the SOS values. Compared with unfixed tissues, formalin fixation induces tissue stiffness due to cross-links among proteins [13]. Regarding the RA, no significant differences in SOS values of the RA were found between FFPE and fresh-frozen sections in 95% ethanol and 10% buffered formalin fixatives [14]. Regarding the aorta, the SOS values in FFPE aortas were greater than those in fresh-frozen aortas, but within the same range [7,16,17]. Second, tissue sections for observation have several limitations. The observation area is 4.8-mm square in maximum due to the restriction of our instrument's scanning area. The section's thickness was approximately 10 μm because no sound can return due to loss of energy from too thick sections and that trapping of two waves from top and bottom of the section is difficult from too thin sections. Irregular-surfaced sections, such as having calcifications or wrinkles, are difficult to measure due to reflection and refraction. As a standard value of SOS, an empty area without tissues is necessary for measurement at the least one corner, where SOS value is 1495 m/s through the water at 25°C.

Plans for future cardiovascular study

Histological observation using SAM can reveal mechanical alterations based on the biochemical abnormality. A study using SAM has shown that mechanical and structural alterations always accompanied biochemical changes of the constituents. ECM components, such as collagens, elastic fibers, vitronectin, fibronectin, fibrillin, matrix metalloproteinases, and lysyl oxidase, are associated with these alterations [7]. Additional analytical modalities, including immunohistochemistry and mass spectrometry, can help find the components.

Conclusion

SAM simultaneously provides structural and mechanical information from the same slide as LM. It supplies objective evidence of mechanical strength because SOS correlates with stiffness. Moreover, comparing SOS values before and after proteinase treatment discloses the susceptibility of the tissues studied to breakdown.

This method is beneficial for cardiovascular studies on atherosclerosis, aging, myocardial infarction, cardiac valve dysfunction, and mechanical rupture to compare the amounts of fibrous substance, such as collagens, elastic fibers, and smooth muscles. Moreover, chemical modification against proteinase digestion is measurable, such as glycation and cross-linking between molecules.

Histological observation using SAM can reveal mechanical alterations based on the biochemical abnormality.

Conflicts of interest

The author has nothing to disclose regarding conflicts of interest concerning this manuscript.

Subjects and ethics

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethical committee of the Hamamatsu University School of Medicine (approval no. 19-180). Because the study used stored autopsy or surgically- removed samples without a link to the patient identity, the need for written consent was waived. All procedures were conducted according to approved guidelines and regulations of the Ethic Committee.

Financial support

This study is supported by Tokai Foundation for Technology, Japan (no. 4 on March 4, 2020).

Acknowledgments

The author thanks T. Moriki, Y. Egawa, Y. Kawabata, and N. Suzuki for their assistance in preparing the histological samples, Dr. K. Kobayashi (Honda Electronics) for his technical support and advice with SAM, and Enago (www.enago.jp) for the English language review.

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