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AN APPROACH FOR VISUALIZATION OF THE INTERACTION BETWEEN COLLAGEN AND ELASTIN IN LOADED HUMAN AORTIC TISSUES

A. Pukaluk[1], H. Wolinski[2,3], C. Viertler[4], P. Regitnig[4], G.A. Holzapfel[1,5], G. Sommer[1]

[1]Institute of Biomechanics, Graz University of Technology, Austria
[2]Institute of Molecular Biosciences, University of Graz, Austria
[3]Field of Excellence BioHealth – University of Graz, Austria
[4]Institute of Pathology, Medical University of Graz, Austria
[5]Department of Structural Engineering, NTNU, Norway

pukaluk@tugraz.at

Abstract. *Knowledge of the interaction between the constituents of loaded aortic tissues is crucial to expand our understanding of load-bearing mechanisms in the aorta. We have therefore developed a procedure that enables simultaneous multi-photon microscopy imaging of collagen and elastin in human aortic tissue during the biaxial extension test. The microscopy images obtained were verified with the results of the histological staining. The mechanical response was also compared with findings from previously performed biaxial extension tests. The proposed pipeline has shown successful and has great potential for structural analysis of human aortic tissue.*

Keywords: *Human aorta, collagen, elastin, biaxial extension test, multi-photon microscopy*

Introduction

The healthy aortic wall consists of three layers, namely the intima, media and adventitia [1]. Each of the layers is characterized by its own structure and function. From a mechanical point of view, the main role is played by the media responsible for the aortic response to loading and the adventitia, which prevents the aorta from overstretching and possible rupture. Both media and adventitia owe their passive mechanical properties mainly to two proteins, namely collagen and elastin. Although the arrangement of these proteins in the aortic layers in the unloaded state has already been described [1,2], little is known about the changes caused by the load. Therefore, this study proposes a method for the efficient visualization of collagen and elastin in loaded aortic tissue.

Methods

The developed procedure was applied to one medial and one adventitial specimen from a non-atherosclerotic and non-aneurysmatic human abdominal aorta (52 yrs old, female). The aorta was received within 24 h of death and frozen at -20°C.

Sample preparation. The aortic tube was thawed at 4°C prior to preparation for testing and imaging. During preparation, all steps were carried out at room temperature and the samples were kept moist with phosphate buffered saline (PBS) at pH 7.4. Loose connective tissue was removed, and the intact aortic tube was cut open in the longitudinal direction. The intimal and adventitial layers were then carefully dissected from the media [3], and square samples measuring 20×20 mm were cut in order to obtain medial and adventitial patches. In addition, adjacent rectangular patches of dimensions of about 4×10 mm were cut for histological examinations. Particular care was taken to ensure that the edges of squares and rectangles match the anatomical longitudinal and circumferential directions of the aorta. The mean thickness of each sample was measured optically [3]. Each square sample was then pierced by four sets of hooks connected by sutures. A set of five hooks was used on each side [4].

Histology. Aortic specimens were embedded in paraffin and cut at 4 µm with the microtome Microm HM 335 (Microm, Walldorf/Baden, Germany). Next, the sections were stained with Picrosirius Red (PSR) to highlight fibrillar collagen and Elastica van Gieson (EvG) to highlight elastin fibers [5] to verify multi-photon microscopy images.

Multi-photon microscopy. The imaging took place at the IMB-Graz Optical Imaging Resource with a tunable picosecond laser (picoEmerald; APE, Berlin, Germany), which was integrated into a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany). The laser was tuned to 880 nm to induce both the second harmonic generation (SHG) signal from collagen and the two-photon excited (TPE) autofluorescence signal from elastin. A two-channel, non-descanned detector (NDD) in epi-mode was used to detect SHG and TPE signals simultaneously (SP 680 nm barrier filter, i.e., excitation light filter; BP 460/50 nm for SHG signal; BP 525/50 nm for TPE signal; beamsplitter RSP 495 for two-channel separation of SHG and TPE signals). *Z*-stacks were acquired with the HCX IRAPO L 25× NA 0.95 water immersion objective with a large working distance of 1.5 mm for imaging the deep tissue and a sampling interval of 0.6×0.6×5.0 µm.

As a compromise between image quality and acquisition time, four-fold line averaging was used to reduce image noise. A coverglass and water as the immersion medium could not be used with samples mounted on the biaxial test device, since the coverglass

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could not be fixed horizontally and the sample quickly soaked up water. Alternatively, an aqueous eye gel Lac®-Ophtal® Gel (Dr. Winzer Pharma, Berlin, Germany) was used [6], and the lens was dipped directly into the gel.

Biaxial extension test. In order to carry out the planar biaxial extension test and the multi-photon imaging simultaneously, a biaxial testing device was constructed, which could be placed on the microscope stage, based on the design described in [7], but limited by the geometrical and environmental requirements of the microscope. The device integrates four high precision linear positioners SLC-2640 (SmarAct, Oldenburg, Germany) with the maximum travel range of 35 mm and 1 nm resolution while the maximum velocity is limited to 20 mm/s and the maximum blocking force to 3.5 N. Each positioner carries a bracket with an assembled load cell KM10z 25N (ME-Meßsysteme, Hennigsdorf, Germany) characterized by a maximum permissible force of 25 N and 1% accuracy class. The design of the device allows displacement and force measurements in two perpendicular directions (Fig. 1) with one set of sensors on each side.

The stretch-driven testing protocol was implemented with the LabView software (National Instruments, Austin, USA). All samples were loaded equibiaxially and quasi-statically at a speed of 3 mm/min. First, a sample was subjected to the pre-load of 10 mN, which was defined as the reference configuration at a stretch of 1. The pre-load was followed by cycles of preconditioning to obtain a reproducible response. The *z*-stack series of images was then taken in the center of the sample. After imaging was completed, the sample was stretched to 1.02 and imaged again. The experiment was repeated with 0.02 stretch increments until the stretch of 1.40 was reached.

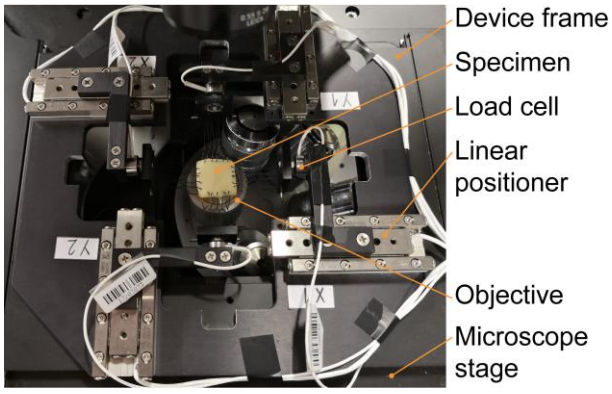

Figure 1: Biaxial testing device with a hooked specimen placed on the microscope stage.

Results

Histology. For the media, the histological staining showed crimpy collagen fibers embedded in a network-like arrangement of elastin fibers (Fig. 2). In contrast, the adventitia showed smooth, wavy collagen bundles accompanied by separate, either curly or straight, elastin fibers.

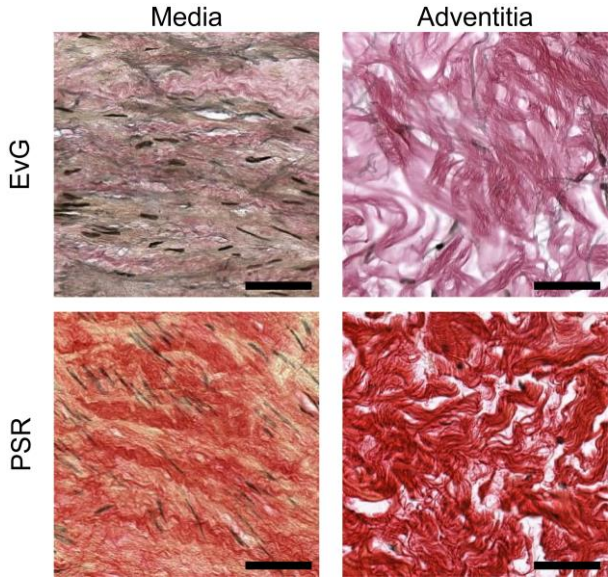

Figure 2: Histological staining of the aortic layers; scale bars denote 50 µm.

Multi-photon microscopy. The emission signal transmitted through the BP 460/50 nm and BP 525/50 nm filters was color-coded in green and red, respectively (Fig. 3). The red-colored channel, which was expected to reflect elastin, captured the network-like fibrillary structure in the media and individual fibers in the adventitia.

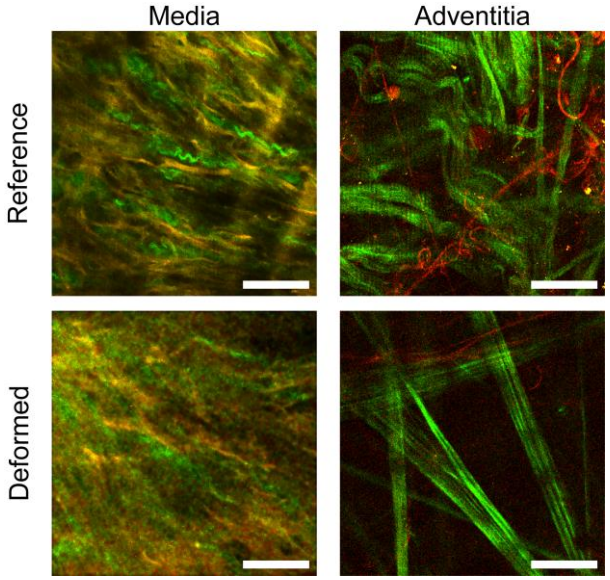

Figure 3: Multi-photon microscopy images of aortic layers at the reference and deformed (max stretch achieved) configurations; scale bars denote 50 µm.

Both images resembled the elastin shown by the EvG staining (Fig. 2). For the media, however, the green-colored channel contained not only the curly fibers corresponding to the histological analysis, but also the network-like structure of elastin. The spectral crosstalk of SHG signal from medial collagen and the TPE signal from elastin was observed as a yellow color in the merged images (Fig. 3). For adventitia, the green-colored channel contained fiber bundles

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that were comparable to adventitial collagen as stained by EvG and PSR.

Biaxial extension test. The experiment was successfully carried out up to a stretch of 1.40 for the media, but has to be stopped at a stretch of 1.28 for the adventitia (Fig. 4) due to the overload of the linear positioners. In addition, tissue relaxation was observed as a decrease in Cauchy stress during imaging. Nevertheless, the characteristic mechanical response of both layers was recorded. Adventitia showed a stiffer response and more pronounced anisotropy than the media. In addition, a stiffer longitudinal response was observed for the adventitia while it was observed in the circumferential direction for the media.

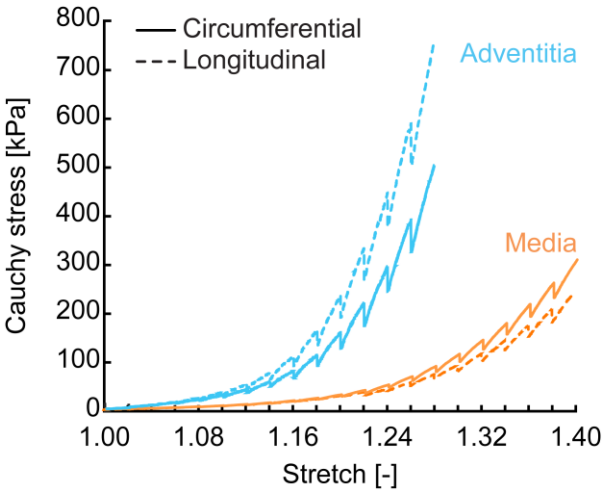

Figure 4: Cauchy stress vs stretch behavior obtained from equibiaxial mechanical tests.

Discussion

Multi-photon microscopy. In the course of this study, the importance of the correct setting of multi-photon microscopy and its validation was demonstrated. Although filters with an equivalent transmission range were used for divergent collagen examinations [8-11], this turned out to be unsuitable for imaging the untreated human aortic media without further processing, e.g., image subtraction.

The SHG signal from collagen can be observed to be induced by the laser excitation wavelength in the range from 730 to 940 nm [12,13]. The excitation wavelength commonly used is around 800 nm [10,14,15] as described by Zoumi et al. [12] or around 880 nm [8,9,11,16], which corresponds to near 900 nm, as reported by Chen et al. [17]. For this study, the emission wavelength 880 nm was chosen based on our previous studies on the human abdominal aorta [8,9], as the signal was observed to be optimal for this tissue in terms of the intensity of the emission. Differences in optimal values of the excitation and emission wavelengths can be caused by the sensitivity of the collagen SHG signal to the biochemical properties of the solution in which the fibrils are located. Although it proved to be insensitive to the pH value within the physiological range, it changes dramatically with the ionic strength of the solution [18].

The spectral crosstalk of medial collagen and elastin identified during this study was also observed for the human thoracic aortic media imaged by Koch et al. [19], who excited the tissue with a laser wavelength of 830 nm and used 400±50 nm and 525±25 nm bandpass filters to capture collagen and elastin, respectively. Interestingly, no prominent spectral crosstalk was reported by Phillippi et al. [20] when examining the human aortic media with the same settings. This discrepancy can be caused by using different gains for the distinct channels. In addition, van Zandvoort et al. [21] observed a spectral crosstalk of elastin TPE in the carotid arteries of mice using 410-490 nm bandpass emission filter. The presence of a TPE signal for elastin in this shorter wavelength range (410-490 nm) can be caused by a relatively higher intensity of this TPE signal compared to the SHG signal of collagen [22].

Biaxial extension test. During the test it was not possible to provide a physiologically similar environment (immersion with PBS at 37°C), resulting in noticeable drying of the tissue borders, which can affect the mechanical response. In addition, the Cauchy stress-stretch curves are affected by relaxation phenomena during imaging. Despite these limitations, our results are comparable with other studies.

Similar to our study, Niestrawska et al. [8] reported higher mean values of the Cauchy stresses in the circumferential than in the longitudinal direction for the medial layer of the human abdominal aorta. For the adventitia, too, a stiffer response in the longitudinal direction compared to the circumferential direction was reported in previous studies [8,23].

Further implications. The presented novel combination of multi-photon microscopy and biaxial extension tests provides an insight into the microstructure of the human aortic layers, which are exposed to increased equibiaxial stretch. The visualized collagen and elastin can be further analyzed and quantified in order to obtain important structural parameters such as orientation, dispersion, thickness and waviness of the fibers. Available material models are not yet able to take into account all of the structural parameters mentioned above. Therefore, combined microstructural and biomechanical data, as provided in the study, are essential to develop and calibrate novel material models to better reproduce and predict the mechanical behavior of aortic tissues in health and disease.

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