Analysis of Heterogeneous Deformation in the Wall of Rabbit Thoracic Aorta at Microscopic Level

Yong Fan,* Junfeng Wang,* Eijiro Maeda,* Kohei Murase,* Takeo Matsumoto*,#

Abstract Aortic wall changes dimensions and mechanical properties in response to mechanical stimulation. As these changes are driven by the cells inside the wall, and their mechanical response has been suggested to exhibit a close correlation with nuclear deformation, it is necessary to study the deformation of the aortic wall at microscopic level. Hence, we obtained 200-µm-thick slices of rabbit thoracic aortas in the circumferential-radial and longitudinal-radial planes, and stretched them in the circumferential and longitudinal directions, respectively, under a microscope. The nuclei of smooth muscle cells (SMCs) were stained with Hoechst33342. Each slice was repeatedly stretched stepwise by 4%, while the fluorescence images of the cell nuclei as well as the elastin auto-fluorescence were captured at each step. Macroscopic and microscopic stretch ratios were obtained from the fluorescence images. Local Green strain was calculated from the change in internuclear distance in a specimen stretched in the circumferential direction. The local tissue strain in the circumferential direction was 0.8 to 2.1 times the macroscopic tissue strain, indicating that the aortic wall deformation was heterogeneous at microscopic level. The shear deformation between adjacent elastic laminas was evident at specific locations, resulting in a shear strain as large as 10%. We also evaluated the relationship between tissue deformation and nuclear deformation from the change in nuclear shape in the specimen stretched in the circumferential and longitudinal directions. In the circumferential stretch, the strain calculated from the length of the nuclei was less than 70% of the macroscopic strain, suggesting that the nuclei of the SMCs are much stiffer than the cytosolic components. Some nuclei rotated noticeably in response to the stretch, and the average and maximum rotation angle was 5° and 11°, respectively, during the entire stretching process. In the longitudinal stretch, the change in nuclear length was not significant, suggesting that mechanical stimulation to the SMCs may be smaller in this direction, as reported previously. The present study shows that the deformations of both the extracellular matrix and cell nuclei are highly heterogeneous, which may have a profound effect on the vascular biology.

Keywords: vascular wall, biomechanics, heterogeneity, mechanical analysis, tensile test.


1. Introduction

Aortic walls are well known to change dimensions and mechanical properties in response to mechanical stimulation. Many studies have reported that the thickness of the aortic wall increases in hypertensive animals to maintain the circumferential stress in the physiological state at the level of normotensive animals [1–5]. In contrast, such adaptations may not occur in the longitudinal direction. Matsumoto and Hayashi [5] reported that the circumferential stress did not change significantly in response to hypertension, while the longitudinal stress decreased significantly by 40% in rats with induced Goldblatt hypertension for 8 weeks. Wall thickening of the aorta is thought to be driven only by the cellular components in the media, i.e., smooth muscle cells (SMCs) [6]. Recently, Nagayama et al. [7] found that chromatin distribution in the nucleus changed significantly in response to mechanical stimulations. Inspired by their results, we propose a new hypothesis of wall thickening. Hypertension increases mechanical stimulation to SMC nuclei, subsequently promoting dispersion of chromatin in the nuclei that possibly stimulates protein synthesis, and eventually causing wall thickening. These changes may be induced by the change

This study was presented at the Symposium on Biomedical Engineering 2018, Nagoya, September, 2018. Received on August 4, 2018; revised on October 31, 2018; accepted on November 6, 2018.

* Biomechanics Laboratory, Department of Mechanical Systems Engineering, Graduate School of Engineering, Nagoya University, Aichi, Japan.
# Furo-cho, Chikusa-ku, Nagoya, Aichi 466–8603, Japan.
E-mail: takeo@nagoya-u.jp

DOI:10.14326/abe.8.7
in cell shape within the wall, thus leading to nuclear deformation. To verify this hypothesis, the relationship between nucleus deformation and arterial wall deformation must be characterized.

The media of the aorta primarily consist of SMCs, elastin fibers, and collagen fibers. The aorta has been demonstrated to show complex mechanical behaviors when subjected to physiological loading from the non-loaded state in vivo [8]. These complex mechanical phenomena are caused by the intricate structure of the three components [9]. The intricate structure also causes the heterogeneity of stress distribution in the media [10]. Furthermore, protein synthesis in SMCs has been suggested to correlate closely with the heterogeneity of the vessel wall [11]. In addition, deformation of the aorta due to pressurization is complicated because of the structural heterogeneity of tissue layers and the differences in elastic properties of the elastin layers (ELs), smooth muscle layers (SMLs), and surrounding collagen and elastin [12]. Nonetheless, the details of heterogeneous deformation and strain field within the media remain to be studied.

The present study was performed to investigate the heterogeneous deformation of the aortic wall and the deformation of the nuclei under tensile stretch. We first performed a circumferential tensile test on the aortic wall, mimicking vessel dilation, to evaluate its deformation at microscopic level. Next, to investigate the difference in mechanical response in the radial and circumferential directions of the aortic wall, particularly the SMC nuclei, the tensile test was performed on sections cut in the direction perpendicular to the longitudinal axis of the blood vessel, or perpendicular to the circumferential direction.

2. Materials and Methods

2.1 Specimens

Thoracic aortas harvested from 17–19-week-old male Japanese white rabbits weighing 3.1–3.5 kg were cut into 200-μm-thick ring-like segments in the direction perpendicular to the longitudinal axis of the aorta, or cut into 200-μm-thick strip-like segments in the direction perpendicular to the circumferential direction using a micro-slicer (DTK-1000, Dosaka EM, Japan), within 3 h after the aorta was harvested from the rabbit, as shown in Fig. 1. All animal experiments were approved by the institutional review board for animal care at the Nagoya University (approval #18-8) and were performed in accordance with the Guide for Animal Experimentation, Nagoya University.

The slices were immersed in Hoechst33342 (Molecular Probes, USA) diluted 1:500 in phosphate buffered saline without calcium and magnesium [PBS(-)] for 1 h at room temperature for staining the SMC nuclei. Each of the 200-μm-thick ring-like segments was cut into a rectangular specimen of approximately 0.3 mm × 7 mm in the circumferential direction. Similarly, the 200-μm-thick strip-like segments were cut into rectangular specimens along the longitudinal axis with a length of 7 mm.

2.2 Tensile test

The experiment was performed on a tensile tester (STB-150W-NK, Strex, Japan) under a confocal laser scanning microscope (IX81+FV1200, Olympus, Japan) at room temperature. The tester was originally designed to stretch cells on an elastic membrane, and was modified to stretch small thin-sliced specimens (Fig. 2). The two ends of the
rectangular specimen were attached to two 0.1-mm-thick OHP sheets of 6 mm × 3 mm using cyanoacrylate adhesive. Each of the OHP sheet was attached on a custom-made stainless steel piece using the same cyanoacrylate adhesive. Next, the pieces with the specimen were mounted to the arms of the tensile tester via silicone jigs. Finally, the tester was mounted to the microscope.

For the stretching experiment, the initial position of the specimen was determined as the nonloaded state that should satisfy the following two conditions. First, a clear fiducial nucleus is located in the center of the field of view, and both the nuclei and ELs can be observed clearly. Second, the wavy morphology of the ELs begins to straighten as the gears of the tester are rotated slightly. Once the initial position was set, a series of Z-stack images of the nuclei and ELs were acquired. Tensile strain was imposed on the specimen stepwise with an increment of approximately 4% in the circumferential or in the longitudinal direction of the specimen by manually rotating the gears on both sides of the tester together. At the end of each straining step, a series of Z-stack images were obtained. We repeated the cycle of strain increment and Z-stack imaging 10 times. Each series of the Z-stack images consisted of 15–20 slices with an interval of 1.5 μm. The duration for an entire single stepwise stretch was approximately 3 minutes. The entire experiment was performed within 48 h after the vessels were harvested.

During the tensile test, fluorescent images of the SMC nuclei were obtained at Ex/Em = 405/430–470 nm using a diode laser LD405 (405 nm, Olympus), while the auto-fluorescence images of the ELs were obtained simultaneously at Ex/Em = 488/505–605 nm excited using a multiline Ar laser FV5-LAMAR-2 (488 nm, Olympus) [Fig. 3(a)]. A 100 × lens (NA = 1.4) was used for observation and photography during the test. PBS(-) was intermittently dripped onto the specimen to prevent dehydration.

### 2.3 Image processing and calculation methods

The ImageJ ver.1.5 (NIH, USA) software was used to process and analyze the nuclei and ELs in all the images acquired. Each series of the Z-stack was projected onto a single image using the standard deviation projection in ImageJ. To obtain the macroscopic stretch ratio in each stretching experiment, we chose three pairs of characteristic points in the image of the initial position. For each pair, one point was located near the left edge of the image, and the other near the right edge of the image. Further, the positions of the three pairs perpendicular to the stretch direction were chosen to be almost equal. The circumferential distance between each pair of points was measured at each strain step. The macroscopic stretch ratio at the nth strain step from the initial position was obtained as

\[
\Lambda_n = \prod_{i=1}^{n} \Lambda_{(i-1)\alpha}
\]

where \(\Lambda_n\) is the macroscopic stretch ratio of the specimen; \(\Lambda_{(i-1)\alpha}\) the stretch ratio between stretch steps \(i-1\) and \(i\) averaged for the three pairs.

For the images obtained from the tensile test in the circumferential direction, we selected the SMLs in the images according to the morphology of the ELs and numbered them [Fig. 3(a)]. Similarly, we chose a pair of characteristic points or centroids of two nuclei with almost equal radial position on the left and right sides of each SML. The change in the circumferential distance between these two points was measured to obtain the stretch ratio in each SML as

---

**Fig. 3**

(a) Fluorescent image

(b) Elongation of each lamina

(c) Shear deformation between laminae

(a) Example of fluorescent image of cell nuclei and elastin laminas. Rectangles indicate SMLs selected for analysis. A-G and a-g correspond to the left and right points in each rectangle. (b) Relationship between the stretch ratio of elastic laminas and the ratio of the macroscopic stretch \(\Lambda\). (c) Relationship between shear deformation between two adjacent laminas and the macroscopic stretch ratio \(\Lambda\). Bar = 30 μm.
\[
\lambda_{mn} = \prod_{i=1}^{n} \frac{L_{mi}}{L_{m(i+1)}}
\]

where \(\lambda_{mn}\) indicates the stretch ratio of the SML \(m\) at the \(n\)th strain step, \(i\) the stretch step, and \(L\) the distance between the two chosen points in each SML.

For calculating the shear deformation between two SMLs, we first calculated the coordinates of the central point between the two side points in each SML selected as described above. Subsequently, we obtained

\[
\tau_{(m)(m+1)} = \frac{|\bar{X}_{(m+1)i} - \bar{X}_{(m+1)i+1} - |\bar{X}_{mi} - \bar{X}_{mi+1}|}{d_{(m+1)i} - d_{mi}}
\]

where \(\tau_{(m)(m+1)}\) is the shear deformation between the SML \(m\) and \(m+1\); \(d_{mi}\) and \(d_{(m+1)i}\) are the radial coordinates of the central point of SML \(m\) and \(m+1\), respectively; \(\bar{X}\) is the mean value of the two chosen points in each SML.

For calculating the local Green strain, we set small triangles in the image of the initial position. Each line of the triangles is always connected to the same characteristic points in the images obtained under tensile strain (Fig. 4 a–c). The local Green strain relative to the initial step 0 was calculated as

\[
ds^2_{(1,2,3)} - ds^2_{(1,2,3)} = \sum_{j=1}^{2} \sum_{k=1}^{2} 2E_{jk}da_j da_k
\]

where \(ds^2\) is the line length of the triangle at the present strain step, \(ds^2\) the line length at the initial step, \(E_{11}\) the local Green strain along the circumferential direction, \(E_{22}\) the local Green strain along the radial direction, and \(E_{12} (= E_{21})\) the shear strain. Further, \(da_j\) and \(da_k\) are the components of \(ds\) in the circumferential and radial directions, respectively. The calculation was performed using a custom-made MATLAB code (Ver. 2016a, Mathworks, USA).

The relationship between tissue deformation and nuclear deformation was also evaluated from the changes in nuclear shape in the specimen stretched in the circumferential and longitudinal directions. To determine the nuclear deformation, we selected several nuclei at various positions in each specimen. The outlines of these nuclei were traced using the Freehand Selections tool in ImageJ. Each outline was fitted to an ellipse for the measurement of the central coordinates, the length of the major axis \(l_e\), the area \(A_e\), and the angle to the horizontal direction. The width of the ellipse (nucleus) \(h\) was calculated as

\[
h = \frac{A_e}{l_e}
\]

Fig. 4 Example of Green strain distribution in the circumferential (a) and radial (b) directions, and the shear component (c). Distribution at \(\lambda = 1.16\) (upper panels) and change in each triangle in response to circumferential stretch (lower panels). a to x in the lower panels correspond to individual triangles in the upper panels. Bars = 30 µm.
3. Results

3.1 Comparison between macroscopic and microscopic stretch ratios in the specimens stretched in the circumferential direction

The representative data of macroscopic elongation of the specimen and microscopic elongation of the SMLs are shown in Fig. 3. The elongation of each layer was relatively close to the homogeneous body line where the macroscopic and microscopic stretch ratios were the same [Fig. 3(b)]. In terms of strain (= stretch ratio - 1), the maximum deviation of the absolute difference between the value of individual layer and the homogeneous body line was 20% at $\lambda = 1.45$. The elongation rates of the SMLs were inconsistent among the seven layers up to $\lambda = 1.16$, while the rates were almost consistent beyond this point. This may indicate that the wavy ELs become almost straight and rotation of most of the nuclei ceases beyond this point, causing homogeneous deformation. The shear strain between two adjacent laminas changed primarily in the low-strain range ($\lambda < 1.16$) and subsequently became stabilized beyond this point except at $\lambda = 1.46$ [Fig. 3(c)]. This may indicate that heterogeneous deformation occurs primarily in the low-strain region, as shown in (b). The maximum shear strain was as large as 10%. Considering that the shear strain in the homogeneous body is zero, 10% strain is not negligible. The results of another specimen showed maximum deviation of 6% for the stretch ratio, and within 12% for shear strain. The mean maximum deviation for the lamina stretch ratio of the two samples was 13%, and the mean maximum deviation for the shear strain was 11%.

A representative example of the Green strain analysis is shown in Fig. 4. When each of the Green strain components; namely $E_{11}$, $E_{22}$, and $E_{12}$, was plotted against the macroscopic stretch ratio $\Lambda$, the deviation from the homogeneous body line was as high as 117% in $E_{11}$. The $E_{11}$ values varied within the same SML as well as between different SMLs [Fig. 4(a)]. Contrary to the shear strain between adjacent layers, $E_{11}$ continued to increase with increase in circumferential stretch. In contrast, $E_{22}$ decreased as the sample was stretched in most cases when compared to the homogeneous body line. In some locations, the shear strain of a triangle was twice as large as that between the adjacent layers. These results indicate that the heterogeneity at the lamellar level becomes larger when observed at cellular level.

3.2 Difference in the deformation between the cell nucleus and its surrounding tissue in specimen stretched in the circumferential and longitudinal directions

The stretch ratio of the nuclear length was smaller than the homogeneous body line in most cases when plotted against the macroscopic stretch ratio $\Lambda$ [Fig. 5(a)]. The mean strain in the nucleus was 68% for the homogeneous case at $\Lambda = 1.46$. The maximum strain was approximately 287% of the minimum strain, indicating the possibility of a large difference in the mechanical stimulation received by each nucleus. With regard to the nuclear width, the changing rate was smaller than that of the homogeneous body line in most cases when plotted against the macroscopic stretch ratio $\Lambda$ [Fig. 5(b)]. The mean strain in the nucleus was approximately 70% of the homogeneous case. The maximum strain was approximately 153% of the minimum strain calculated in absolute value. Most of the nuclei rotated in response to the circumferential stretch and were aligned in the direction of stretch [Fig. 5(c)]. Some of them rotated as much as $11^\circ$–$12^\circ$, with mean rotation angle of 5°.

The nuclear deformation demonstrated a clear dependence on the stretching direction. The mean stretch ratio of the nucleus length increased 1 to 1.3 times during circumferential stretch [Fig. 6(a)] when the specimens were stretched up to 1.46 times, while no significant changes were observed during longitudinal stretch [Fig. 6(c)]. Meanwhile, no such difference was observed in the stretch ratio of the nuclear width.

![Fig. 5](image-url) Stretch ratios of nuclear length (a) and nuclear width (b), and the angle of the major axis of the nucleus to the stretching direction plotted against the macroscopic stretch ratio.
Discussion

In the present study, we focused on the mechanical heterogeneity of the rabbit thoracic aortic wall by measuring the deformations of ELs and SMLs, as well as the nuclei of SMCs during the tensile test in the circumferential and longitudinal directions. The elongation of each SML showed weak heterogeneity in response to circumferential stretch (Fig. 3), and this heterogeneity became more remarkable when the deformation was evaluated for local Green strain (Fig. 4). These results may suggest that the heterogeneity may increase in magnitude when examined in smaller scales.

As shown in Figs. 3(c) and 4(c), the aortic wall showed shear deformation during circumferential stretch. This clearly indicates that the aortic wall is deformed with a combination of stretch and shear deformations during the tensile test, which is consistent with the findings of a past study [8]. Although the amount of shear deformation was not large (<0.1) in most cases, this might exert profound effects on the physiological functions of the SMCs.

The precise mechanism causing such heterogeneities is still unclear at this stage. However, the apparent structural heterogeneity in the wall may account for the observed heterogeneity. This includes the varied thickness and structure of the SMLs and ELs, and the connections of ELs to the collagen fiber networks. Since the ELs and the nuclei can be visualized only in the superficial region (approximately 20 µm from the surface at the maximum) of the 200-µm-thick specimens, a new method that can visualize the specimen in the whole thickness is awaited.

The nuclei showed lower stretch ratios than the homogeneous body [Fig. 5 (a, b)]. This may indicate that the nuclei are stiffer than the cytoplasm, and/or the physical connections between the nuclei and extracellular matrix components are weak. In addition, large variations might be present in the mechanical stimulation exerted on each nucleus as their stretch ratios and rotation angles varied markedly [Fig. 5 (c)].

The SMC nuclei showed larger stretch ratios when stretched in the circumferential direction than in the longitudinal direction (Fig. 6). Such differences in the stretch ratio may be attributed to more actin filaments linking the plasma membrane and the nucleus, and greater distribution along the long axis of the cells than in the radial direction. This could also be the reason for the greater effect of external circumferential stimulation on the cells than stimulation along the longitudinal direction [5]. The cells, or more specifically, the stress fiber in the cells are in a fully relaxed state because the test was performed in PBS(-) at room temperature. Since the deformation of the cell body is conveyed to the nuclei via the stress fibers, the stretch ratio of the nuclei might be lower than the physiological condition.

This study has several limitations. First, the experiments were performed at the room temperature. As temperature strongly affects the activity of SMCs, the SMCs are supposed to lose their physiological responsiveness to mechanical environment in the current experimental setting. Further experiments should be performed with the temperature maintained at 37°C. Next, we only performed the analyses on two-dimensional images projected from three-dimensional (3-D) image stacks. As the aorta has a 3-D structure, we need to perform a 3-D deformation analysis using 3-D image reconstruction. This may be especially important when measuring intranuclear distance because the nuclei are expected to show complicated 3D movements. Finally, we only evaluated the circumferential deformation of sliced tissue specimens. As the blood vessels are tubular, constraints exist regarding observation of deformation in the longitudinal direction. To observe the deformation of the internal structure of blood vessel while maintaining the physiological boundary conditions, advanced imaging techniques such as two-photon microscopy may be useful.

Conclusion

In conclusion, the SMLs in rabbit thoracic aorta exhibited heterogeneous deformation in response to circumferential tensile stretch at both macroscopic and microscopic levels, and the heterogeneity was more prominent at microscopic level. In addition, the deformation of the SMC nuclei was heterogeneous, and their deformation in response to longitudinal stretch were much smaller com-
pared to circumferential stretch. This may explain the relative insensitivity of the SMCs to mechanical stimulations in the longitudinal direction [5].

Acknowledgement

This work was supported in part by KAKENHIs from JSPS (Nos. 15H02209, 15H05860, and 18H03752) and AMED-CREST from the Japan Agency for Medical Research and Development (17gm081005h0002).

References


Yong Fan

Yong Fan received his master degree in Mechanical Engineering from Tianjin University of Technology (China) in 2016. He is presently a Ph.D. student at Department of Mechanical Systems Engineering, Graduate School of Engineering, Nagoya University. His research interest lies in cardiovascular biomechanics. He is a member of the Japan Society of Mechanical Engineers (JSME).

Junfeng Wang

Junfeng Wang received his Ph.D. degree in Nanopharmaceutical Sciences of Nagoya Institute of Technology in 2016. He is presently a postdoctoral researcher at Graduate School of Engineering, Nagoya University. His research interests are cell biomechanics and biomedical engineering. He is a member of the Japan Society of Mechanical Engineers (JSME) and Japanese Society for Medical and Biological Engineering (JSMBE).

Eijiro Maeda

Eijiro Maeda received Ph.D. degree from University of London in 2008 and has been an assistant professor in Graduate School of Engineering, Nagoya University since 2016. His research interests lie in biomechanics of cell and tissue, in particular how cells and tissues respond to mechanical environment and how that relates to the maintenance of health or the development of diseases.

Kohei Murase

Kohei Murase received his Ph.D. in Engineering from Kyoto University in 1997. He is presently an associate professor at Graduate School of Engineering, Nagoya University. His research interests are biomechanics simulations and biomedical engineering. He is a member of the Japan Society of Mechanical Engineers (JSME) and Japan Society for Clinical Biomechanics.

Takeo Matsumoto

Takeo Matsumoto received his Ph.D. in Engineering from Hokkaido University in 1988. He is presently a professor at Department of Mechanical Systems Engineering, Graduate School of Engineering, Nagoya University. His research interests include biomechanics, mechanobiology, and biomedical engineering. He is a member of various academic societies including Japanese Society for Medical and Biological Engineering, Japanese Society of Bioengineering, the Society of Life Support Engineering, Biomedical Engineering Society, and the American Society of Mechanical Engineers, a fellow of the Japan Society of Mechanical Engineers, and currently the president of Asian-Pacific Association for Biomechanics.