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Original Article

Regional variations in canine descending aortic tissue mechanical properties change with formalin fixation

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Abstract

Background/introduction: Diseases of the aorta can alter the local mechanical properties of the tissue, leading to aneurysms and plaque instability. Local tissue properties are best evaluated from surgical samples or autopsy tissue using mechanical testing ex vivo. We examined whether formalin-fixed tissues preserve regional and local variations in tissue properties when compared to fresh tissues in order to determine if fixed tissue can be used to infer mechanical changes associated with tissue remodeling. **Methods:** Equibiaxial mechanical tests were performed on canine descending thoracic aorta to quantify the regional and local tissue stiffness. Samples were taken from four locations along the aorta and from the lateral and medial side at each location. Half of the samples were randomly formalin fixed and used to measure the effect of fixation on local thickness, stiffness, and anisotropy. **Results:** In fresh tissue, regional differences in tissue stiffness and thickness are present. Aortic tissue stiffens and thins along the aorta. Fixation did not alter thickness, significantly increased tissue stiffness, and altered the directional dependency of the mechanical properties (anisotropy) at low strain. Formalin fixation altered local stiffness of the aorta near the aortic arch. **Conclusion:** The changes in mechanical properties along the aorta were preserved in formalin-fixed samples. However, our results show that formalin fixation can change the variation in tissue stiffness and significantly affects the anisotropic properties of vascular tissues. Formalin fixation introduces spurious changes in mechanical properties, and we therefore strongly recommend the use of fresh aortic tissues for biomechanical analysis.

Keywords: Formalin; Fixation; Descending aorta; Mechanical properties; Canine; Anisotropy; Collagen; Elastin; Stiffness; Biaxial testing

1. Introduction

Cardiovascular diseases such as aneurysms [1] and atherosclerosis [2] are known to occur in focal locations in the human vasculature. The forces created by blood flow are hypothesized to cause structural remodeling, stiffening, and weakening of the tissue. Much research has linked the altered fluid and tissue stresses created by disturbed blood flow with vascular structural and molecular changes [3–9]. Moreover, the structure and composition of the aorta are known to vary along its length [10–12]. Studies have shown that the elastin content decreases along the length of the aorta [13], whereas the amount of collagen is relatively constant [14,15]. This structural difference and increased radius/thickness ratio cause the distal aortic tissue to be stiffer than proximal aortic tissue [15,16].

The identification of regions of local vessel remodeling has become an important observation in the study of the pathogenesis of these diseases. In vivo measurements of the

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elastic modulus and distensibility of the aortic tissue are now common clinical measures from medical imaging devices such as computed tomography, magnetic resonance, and ultrasound [17–21]. However, these estimates can only predict the global isotropic mechanical properties within the measured physiologic range [19,21]. In order to better characterize the local remodeling and mechanical behavior of aortic tissues, ex vivo testing has been used, requiring a large number of samples to draw meaningful conclusions [22–24]. Fresh tissues have been relied upon for performing such local mechanical analysis [22]. When fresh tissue is readily available and if it is possible to test it in a timely fashion, then it should be used.

Understanding the regional changes in the mechanical properties of the aorta is fundamental to better prediction and treatment of aortic disease. Unfortunately, this type of analysis is limited by the ability to collect and test fresh tissue samples in a timely manner. The present study investigates if formalin-fixed aortic tissues can be reliably used to infer regional and local changes in the mechanical properties of the aorta.

2. Materials and methods

2.1. Animals

All animal tissues were collected in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Publication No. 8523, revised 1996). The local institutional ethical committee on animal care approved all procedures. Mongrel dogs (N=16, 27.4±5.8 kg) were anesthetized with pentobarbital sodium (30 mg/kg), isoflurane (1.25%), ketamine (500 mg), and xylazine (200 mg). The descending thoracic aortas were removed from the animals as one continuous piece up to the renal arteries (Fig. 1A).

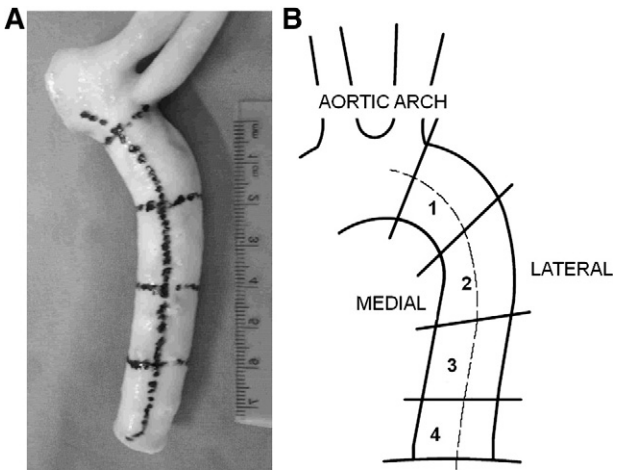

Fig. 1. (A) Photographs of canine thoracic descending aorta sections showing the regions taken for analysis. (B) Drawing indicating the different regions which were taken for analysis.

2.2. Sample preparation

The excised aortic tissue was immersed immediately in Krebs-Ringer bicarbonate buffer (Sigma Aldrich, K4002) until processed. Eight aortic samples were taken from each aorta, identified by its region number (1–4) and location (lateral or medial) (Fig. 1B). The vessel was cut open along the posterior intercostals arteries, leaving the outer curvature (lateral side) on the right side and the inner curvature (medial side) on the left side. One sample of 15 mm by 15 mm was cut from each of the sections with the edges aligned with the circumferential and axial directions of the aorta. The circumferential and axial directions were identified, and the samples were stored in Krebs-Ringer bicarbonate buffer or 10% buffered formalin (Fisher Scientific, F5900). After 24 h, the tissues were rinsed and stored in Krebs-Ringer bicarbonate buffer for an additional 24 h at 4°C. The width and length were measured at three locations along each specimen using dial calipers (Digimatic Calipers, ±0.02 mm), and the thickness was measured using a Mitutoyo apparatus (Litematic VL-50A/VL, accuracy using a constant force, 0.01 N).

2.3. Biaxial mechanical testing

Equibiaxial mechanical tests were performed using an EnduraTEC ELF 3200 biaxial tensile tester system (Bose Corporation, MN, USA) (Fig. 2A). A video extensometer system was not used to monitor the strain field and compute the Green strain tensor during biaxial stretching [25,26]. Although the distance between the sutures represents an approximation of the strain field within the tissue, as the deformation in biaxial tensile testing is coupled between the two orthogonal axes, resulting in a nonuniform strain field, we believe that it can allow the determination of regional differences in mechanical tissue properties. Thus, instead of using the engineering strain, to highlight that we imposed the same biaxial stretch in both directions proportional to the distance between sutures, a working strain as described below was used.

Samples were sutured with 3-0 thread (0.2-mm diameter) using pledgets to hold the specimen in place, inserted in the mechanical tester, and immersed in Krebs-Ringer bicarbonate buffer kept at 37°C during the tests (Fig. 2B). They were minimally stretched in a straight position using a force of 0.10 N. The gauge length was measured as being the distance between the sutures (8–10 mm). Each test consisted of 10 cycles of preconditioning from 0% to 100% strain based on the distance between the sutures at a constant displacement rate of 5 mm/s, and one test cycle after the sample was preconditioned.

2.4. Analysis

From the force-displacement data collected, the engineering stress and working strain curves were calculated. The

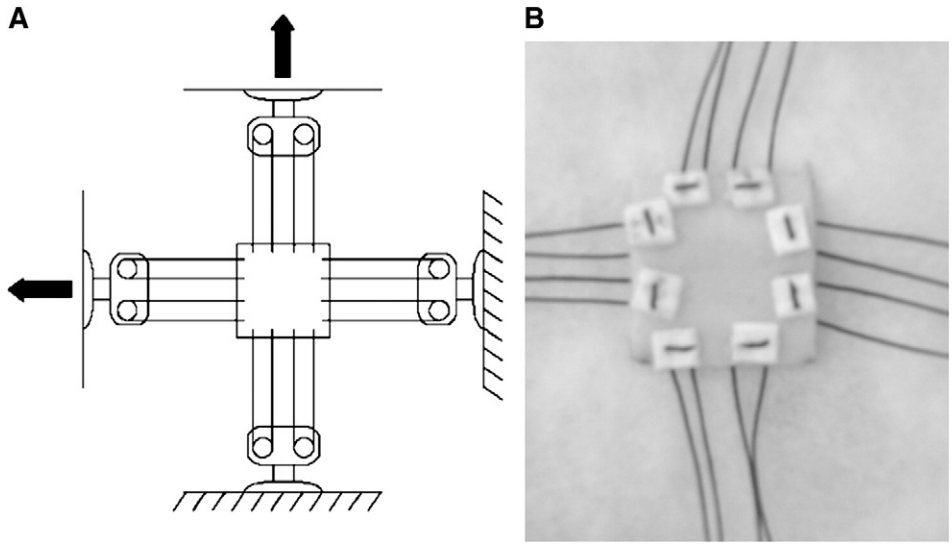

Fig. 2. (A) Schematic of the biaxial mechanical tests. (B) Picture showing the sutures and pledgets used to attach the samples to the biaxial tester.

working strain is defined as the displacement over the initial distance between the sutures, whereas the engineering stress is defined as the force as measured by the load cell over the unloaded cross-sectional area measured as the unloaded width times the unloaded thickness. We chose to work with the engineering stress, which uses the unloaded dimension of tissues, since we could not measure the real tissue strain field to compute the loaded dimension of tissues.

2.5. Incremental elastic modulus

The linear regression toolbox from MATLAB was used to compute the incremental elastic modulus (stiffness) from the stress–strain curves at low and high strains for both directions [27–30]. The slope that best fit the experimental data for working strains comprised between 0% and 60% represents the low-strain stiffness (E_L), whereas the higher range between 90% and 100% represents the high-strain stiffness (E_H). These two regions were identified as linear regions of the loading curve, similar to the technique used by Vorp et al. in the investigation of aortic aneurysms [24] and in studies concerning human ascending aorta tissues [22,31,32].

2.6. Anisotropic index

The anisotropic index (AI) was used to quantify the directional dependency of aortic tissue mechanical properties. It compares the stiffness value in the axial direction to the circumferential direction. This index has been previously used by Lee et al. [33], approaches zero for an isotropic material (no directional dependency), and is defined as:

$$AI = \frac{2(E_{circum} - E_{axial})}{(E_{circum} + E_{axial})}$$

2.7. Fixation ratio

The fixation ratio (FR) was calculated to illustrate the effect of fixation on the stiffness of the tissue at each location. It is the ratio of the difference between the stiffness of fixed and fresh samples over the average stiffness:

$$FR = \frac{2\left(E_{fixed} - E_{fresh}\right)}{\left(E_{fixed} + E_{fresh}\right)}$$

2.8. Statistics

Statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA). All statistics are presented as mean values±standard deviation. One-way and two-way analyses of variance (ANOVAs) were used with Bonferroni multiple-comparisons posttest to identify which groups were different with $P<.05$ considered statistically significant, whereas unpaired two-tailed t tests were used to test the AI and the FR.

3. Results

3.1. Thickness

No difference in thickness was observed between fixed and fresh samples ($P>.05$, two-way ANOVA). In region 1, near the aortic arch, a significant difference between the lateral (outer curvature) and medial (inner curvature) quadrant thickness was observed ($P<.05$, two-way ANOVA), whereas in other regions, no significant difference was observed for both fixed and fresh samples (Fig. 3). Aorta thickness was greater near the aortic arch, and a significant difference was found between the proximal (region 1) and the distal region (region 4; $P<.05$ and $P<.001$ for lateral and medial samples, respectively; two-way ANOVA).

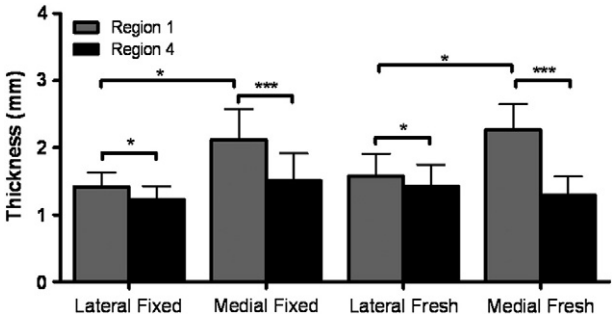

Fig. 3. Histogram showing the thickness in the different regions characterized (regions 1 and 4 are shown) for samples located in the medial and lateral sides. No significant differences were observed between fresh and fixed samples (*P<.05 and ***P<.001, two-way ANOVA).

3.2. Incremental elastic modulus

The low-strain and high-strain incremental elastic moduli (E_L and E_H, respectively) were obtained from the individual stress–strain curves and used for statistical comparison. The magnitude of the elastic moduli of the fixed samples was significantly greater than that the fresh samples at low (E_L) and high strains (E_H) (P<.001 and P<.01, respectively; two-way ANOVA) (Fig. 4). At low strain, the distal portion of the descending thoracic aorta (region 4) was stiffer, although not significantly, than the region close to the aortic arch (region 1). At high strain, this trend was significant, with stiffer tissue in the region distal to the aortic arch (region 4) compared to the proximal section (region 1) (P<.05, two-way ANOVA). These differences (in both E_L and E_H) were preserved after fixation. The difference between the low- and high-strain stiffness is significant, with stiffer tissue at higher strains, for both fresh and fixed tissue.

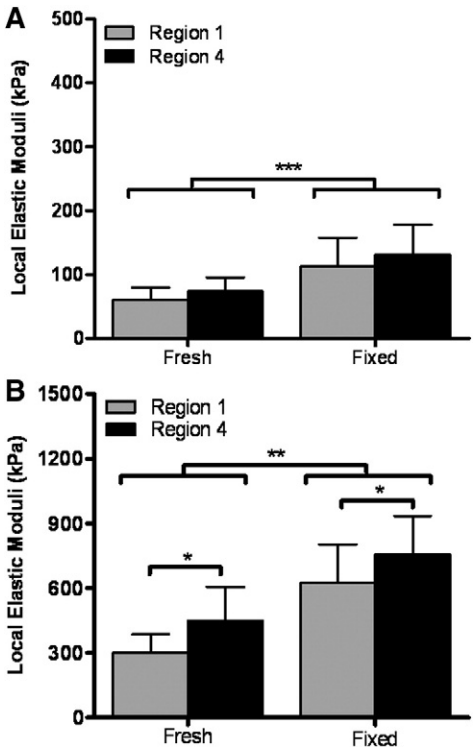

Fig. 4. Combined axial and circumferential local elastic moduli in different regions (regions 1 and 4) and conditions (fresh or formalin fixed) in both the (A) low- and (B) high-strain regions of the engineering stress–strain curve (*P<.05, **P<.01, and ***P<.001; two-way ANOVA).

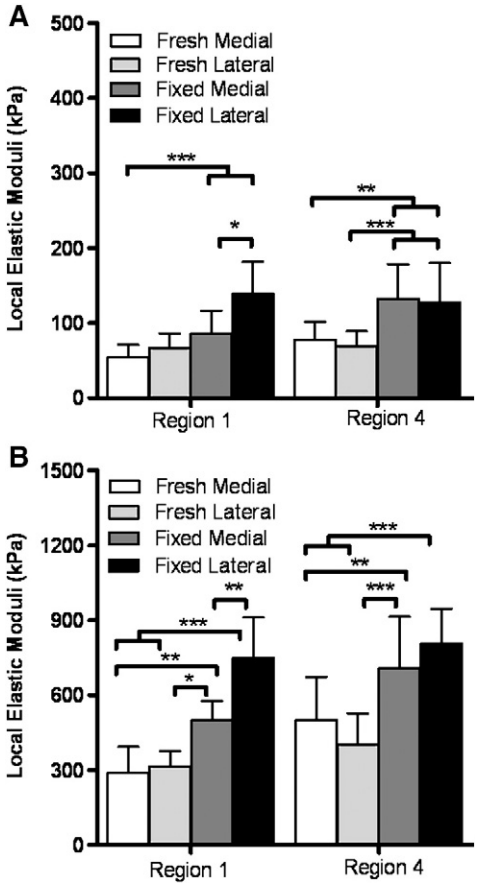

Fig. 5. Local elastic moduli for fixed and fresh samples from the medial and lateral sides in different regions. (A) Low and (B) high strain (*P<.05, **P<.01, and ***P<.001; two-way ANOVA).

No significant differences in the stiffness between the tissue from the medial (inner curvature) and lateral (outer curvature) region were observed in the fresh tissue samples (Fig. 5). Fixing the tissue caused lateral (outer curvature) tissue in region 1 to be significantly stiffer than the medial tissue at both low and high strain (P<.05 and P<.01, respectively; two-way ANOVA).

3.3. Anisotropy index

Slightly positive values of the AI were found for fresh samples at low strain, reflecting that the circumferential direction was slightly stiffer than the axial direction. Fixation significantly changed the AI at low strain (P<.01, unpaired two-tailed t test) by stiffening the tissue in the axial direction after fixation, resulting in negative AI values (Fig. 6). At high strain near the aortic arch (region 1), both fresh and fixed samples were found to be anisotropic (P<.01 and

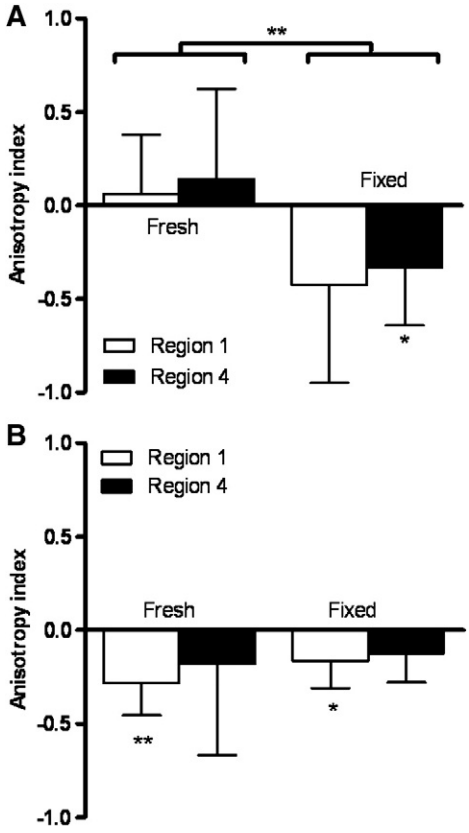

Fig. 6. Anisotropy index, representing the difference between the local elastic moduli in the circumferential and axial direction over their average, in the (A) low- and (B) high-strain regions of the stress–strain curve (*P<.05 and **P<.01, unpaired two-tailed t tests).

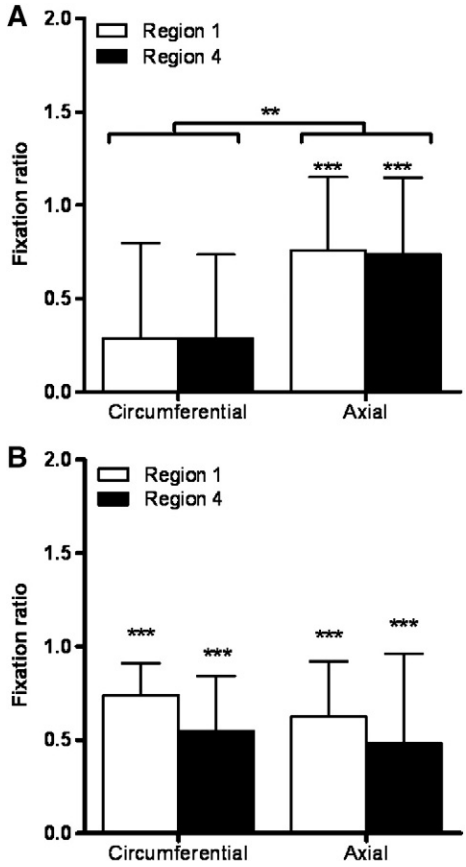

Fig. 7. Fixation ratio in the (A) low- and (B) high-strain regions (**P<.01 and ***P<.001, unpaired two-tailed t tests).

P<.05, unpaired two-tailed t test), being stiffer in the axial direction than in the circumferential direction. Hence, fixation did not alter the AI in the higher strain region.

3.4. Fixation ratio

The FR showed that a significant increase in stiffness occurred in all locations with fixation (P<.001, unpaired two-tailed t test). Interestingly, at low strain, fixation had the greatest effect on the axial stiffness (Fig. 7), which helps to explain the reversal of the AI at low strain (Fig. 6). At high strain, fixation affected the axial and circumferential stiffness equally.

4. Discussion

Formalin fixation helps to preserve tissues, allowing for long-term storage and analysis. Although it is widely used in histopathology research, few studies have examined its effect on tissue mechanical properties [33–35]. Cardiovascular diseases are commonly associated with regional or local mechanical property changes. When studying the biomechanics of vascular tissues, often, the magnitude of the mechanical property is secondary to the identification of the regional and local variations in the mechanical properties. We therefore sought to identify if formalin fixation preserved the regional and local differences in tissue mechanical properties with a hope of increasing the number of samples available for tissue biomechanical testing. Our data confirm that the mechanical properties of the canine descending thoracic aorta are heterogeneous along the length of the aorta. We demonstrated that fixation increases tissue stiffness significantly and preserves the difference in stiffness known to exist between the proximal and distal aorta. Unfortunately, formalin fixation alters the directional dependency (anisotropy) of canine aortic tissue at low strain, rendering the tissue stiffer in the axial direction when compared to the circumferential direction. Fixation also introduced spurious differences in the local stiffness around the circumference of the proximal aorta.

Formalin, a mixture of formaldehyde, water, and salts, forms cross-links between polysaccharides, proteins, glycoproteins, and nucleic acids [36]. Cross-linking alters functional groups in the tissue affecting the solubility and reactivity of proteins [37,38]. Previous studies have shown the effect of fixation with formalin and other fixatives on the mechanical properties of vascular tissue and concluded that fixation can shrink and stiffen tissue [33,39,41,40 36]. We found no changes in tissue thickness due to fixation. Others

have shown formalin to shrink tissue even when the tissue is fixed under pressure [36,42]. Unlike these previous studies, we chose to rehydrate our samples in Krebs-Ringer bicarbonate buffer for 24 h before the thickness was measured, allowing water molecules to enter in the tissue after fixation, possibly explaining why no changes in thickness were observed after fixation (Fig. 2). We found that this preserved the local thickness difference near the aortic arch between the medial and the lateral side after fixation. Fixation also preserved the significant regional difference in thickness between tissue taken near the arch (region 1) and from the lower thoracic descending aorta (region 4), which has been previously observed in multiple species [15,16,43,44].

We found that tissue fixation increased stiffness significantly. Others have also found that fixation increased tissue stiffness of blood vessels [45], valve leaflet [46], and pericardium [32,33]. The increase in stiffness with formalin fixation is attributed to the cross-linking of the structural proteins in the tissue [47]. Glutaraldehyde and formalin fixatives have been widely used to modify the mechanical properties of pericardial tissue used for the fabrication of aortic valve replacement by reinforcing the structure [33,41]. Fixed pericardial tissues are widely used during aortic replacement surgery as a patching material when gaps and tissue weaknesses need to be patched or reinforced [32]. However, to our knowledge, no study has investigated the effect of formalin or glutaraldehyde fixation on the local mechanical properties of vascular tissues. It has been previously shown that formaldehyde and glutaraldehyde both increase pericardial tissue stiffness [46] as we observed in this study with vascular tissues. Also, studies have shown that glutaraldehyde fixation preserved the anisotropic properties of bovine [48] and human [32] pericardial tissues but none in the formalin-fixed vascular tissues we tested. These results are in agreement with our findings regarding the increase in stiffness from fixation.

Regional differences in tissue stiffness exist along the aorta. Several studies have observed increased tensile strength of the aortic wall with increasing distance from the heart from both in vitro and in situ measurements in animals [49–52] and humans [44,53]. Others have shown that the amount of elastic recoil of the aorta after excision varies along the length of the aorta [43,44]. The variation in mechanical properties along the aorta is partly due to changes in the tissue composition. The elastin content decreases along the length of the aorta [13], whereas the amount of collagen is rather stable [14,15], potentially stiffening the artery away from the heart. We did not observe a significant difference in the low-strain stiffness between regions 1 and 4 (Fig. 4). At this strain, elastin is believed to dominate the mechanical response [54]. The increase in stiffness at higher strains along the aorta could be explained again by the changes in the collagen content. At higher strains, collagen is believed to contribute the most to tissue stiffness [55,56]. Overall, the inherent differences in tissue stiffness of the proximal and distal aorta were preserved in formalin-fixed tissues.

Local differences in stiffness between medial and lateral samples were affected by fixation. There was no significant difference between the medial and lateral samples in fresh samples; however, fixation had a significant effect on this local variation in the region near the aortic arch, region 1. This leads to the hypothesis that differences in the composition or arrangement of the structural proteins present in these regions contribute differently to the mechanical properties in response to fixation. It has been shown that elastin and collagen are differently affected by formalin fixation [35,57,58] and that local variations of the structural protein content and orientation exist in curved vasculature [59] similar to region 1. The combination of such behavior could explain the local variation in stiffness we found.

The difference in structural protein orientation can lead to differences in the directional dependency of the mechanical properties. The directional dependency of the aorta has been debated. Some assume that the aortic wall is equally stiff in both the axial and circumferential direction (isotropic) [24,53,60,61], whereas others believe that this is not the case (anisotropic) [45,62]. In this study, we found that the canine descending thoracic aorta was nearly isotropic for fresh tissues at low strain (AI [anisotropy index]~0, Fig. 6), with the circumferential stiffness slightly greater than the axial stiffness. Fixation changed the anisotropic properties of the tissue at low strain, inverting and increasing the anisotropic properties. This phenomenon is likely due to the cross-linking between collagen fibers caused by fixation. These fibers are primarily oriented circumferentially [63], and cross-linking would potentially increase axial stiffness, explaining the negative AI and suggesting that cross-links could contribute more to tissue stiffness than elastin at low strain. This is supported by the FR data (Fig. 7). At low strain, fixation affects the axial stiffness more than the circumferential stiffness. At greater strain, we found the anisotropy ratio of both fresh and fixed tissue to be negative, indicating that the axial was greater than the circumferential stiffness, an observation similar to that of Waldman and Lee [64]. Fixation appears to affect the stiffness equally in the circumferential and axial direction at high strain (Fig. 7).

As in any experimentation, inherent experimental errors and limitations are present. Passive equibiaxial mechanical tests were conducted past the physiological range at a single strain rate, all of which affect the results concerning the mechanical properties of viscoelastic materials [60]. The changes in tissue thickness and the Green strain were also neglected due to limitations in recording strain in real time as opposed to the applied strain [65,66]. However, this does not affect the results that were meant to be comparative. Also, samples were sutured which can also induce variations in the mechanical behavior [64] due to stress concentration near the sutures.

In conclusion, we found formalin fixation to preserve the regional differences in tissue thickness while increasing

tissue stiffness. The regional increase in stiffness along the aorta was preserved with formalin fixation; however, fixation did have a significant effect on the local (medial vs. lateral) stiffness and the mechanical directional dependency of the tissue. These findings do not support the use of fixed vascular tissues for the comparison of local biomechanical properties, and therefore, the use of fresh tissues is strongly recommended.

5. Summary

This study examines the possibility of using formalin-fixed aortic tissues to investigate variations in the regional and local mechanical properties of the aorta. Fixation preserves regional variations in stiffness and thickness along the aorta, but not local stiffness or anisotropic properties of the tissue.

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