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Changes in the Mechanical and Biochemical Properties of Aortic Tissue due to Cold Storage

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Background. **Temporary cold storage is a common procedure for preserving tissues for a short time before using them in a clinical or experimental setting. The process of storing tissues at refrigeration or freezing temperatures can affect the mechanical properties of the tissue. Previous studies were limited to uniaxial tensile tests and did not have substantial explanations for what was structurally occurring in the tissue to account for the changes in mechanical properties.**

Materials and Methods. **This study involved biaxial tensile testing of bovine thoracic aortas that had been stored at common storage temperatures (4°C, –20°C, or –80°C) for three different time points (48 h, 1 wk, or 3 wk). The slopes of the initial and stiff region of the stress-strain curves were measured. The knee point of the stress-strain curves was also determined. Collagen content before and after storage was quantified using a Sircol collagen assay kit.**

Results. **The stored arteries showed decreased initial slope and increased stiff slope after 48 h of 4°C refrigeration. The −20°C and −80°C storage conditions had similar initial slopes compared with the fresh ones but an increase in the stiff slope. There was also a significant shift of the knee point to a higher strain and stress. The soluble and insoluble collagen content decreased significantly due to storage but the percentage of cross-linked collagen was unchanged.**

Conclusions. **Cold storage causes several changes in the mechanical properties due to structural and biochemical changes in the tissue. Overall, freezing protocols (either −20°C or −80°C) are suggested over refrigeration (4°C) for maintaining the initial stress-strain behavior.** **Published by Elsevier Inc.**

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Key Words: **biaxial tensile testing; collagen; mechanical properties; arteries; cold storage; soft tissue; mechanical testing; vascular.**

INTRODUCTION

Understanding the effects of storage conditions on preserving the mechanical properties of artery tissue has both clinical and experimental significance. Allograft material is often used in cardiovascular surgery to repair defects due to cardiovascular diseases or to replace an infected vascular graph. These tissues are often stored with cold storage/freezing protocols before being used [1–8]. Likewise, in laboratory experiments involving human or animal tissues, the samples are usually stored temporarily before testing.

Tissue has been stored in different mediums such as Celsior, EuroCollins, or Ringers solution in order to minimize the effects of storage on the mechanical and biochemical behavior of the tissue [9–11]. Cryopreservation agents such as dimethyl sulfoxide (DMSO) have been investigated with the goal of preserving cells and extracellular matrix during freezing [6, 7]. The use of cryopreserved allografts in treating infections of major vascular prosthetic or peripheral bypass grafts is an area of great interest to cardiovascular surgeons [5, 12, 13]. While cryopreservation has benefits for long-term storage and clinical uses, there is complexity involved with the controlled rate freezing process and the application of DMSO.

Common lab technique involves fresh storage at 4°C and freezing at –20°C or –80°C. During temporary cold storage, there could be a number of causes for changes in the mechanical properties of arteries.

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Arteries are complex structures where the extracellular matrix (ECM) bears the vast majority of wall stress [14]. While the collagen and elastin network is generally responsible for the passive mechanics of the artery, the smooth muscle cells provide the active contraction to facilitate blood flow [15–17]. Cold storage can result in bulk redistribution of water, damage to the collagen network from ice crystals, and breaking of cross-links in the ECM [9, 18–21]. Freezing temperatures can also damage smooth muscle cell functionality or cause them to be completely lysed [6, 7]. These various changes in structure will negatively affect the mechanical behavior of the artery.

The mechanical properties of biological tissues stored at various temperatures have been studied with uniaxial tensile and ring stretching tests in order to determine the Young's modulus of the tissue [8, 19]. Uniaxial testing provides useful information on isotropic materials but is insufficient to investigate the structural and mechanical anisotropy seen in blood vessels. Biaxial tensile testing has been shown to be more effective in fully characterizing the mechanical properties of soft biological tissue [22–27].

In this study, biaxial tensile testing was used to measure the mechanical properties of arteries before and after storage. Storage conditions at 4°C, –20°C, and –80°C were examined in order to study storage temperatures that would be most commonly available for use in a lab. Lactated Ringer's solution was chosen as the storage medium since it is isotonic with blood and is commonly used for temporary preservation of tissue. The length of storage in this study was either 48 h, 1 wk, or 3 wk. Finally, collagen assays were performed in order to quantify the collagen content in the tissues and relate the change in mechanical properties of arteries with their microstructure.

MATERIALS AND METHODS

Sample Preparation

Young bovine descending thoracic aortas were supplied by a local slaughter house and transported to the lab on ice. Upon arrival to the lab, vessels were rinsed with deionized water and loose connective and fatty tissue was removed. Areas of the artery that were approximately 1.5 mm thick were sectioned into 2.0 cm squares (approximately 36 total squares from nine arteries). The squares were cut so one edge was parallel to the length of the artery while the other edge was parallel to the circumference of the artery. The samples were tested with the biaxial tensile tester at the fresh condition within 4 h of receiving the arteries from the slaughterhouse. Details of the mechanical testing procedure are described in the section below. After mechanical testing at the fresh condition, the samples were submerged in lactated Ringer's solution (all chemicals were from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise specified) in 60 mm Petri dishes. The arteries were then either placed in 4°C, –20°C, or –80°C storage freezers for 48 h, 1 wk, or 3 wk. After storage, the samples were thawed for about 1 h at room temperature prior to mechanical testing. The samples were retested so that the data from the stored sample could be compared with the same sample when it was fresh.

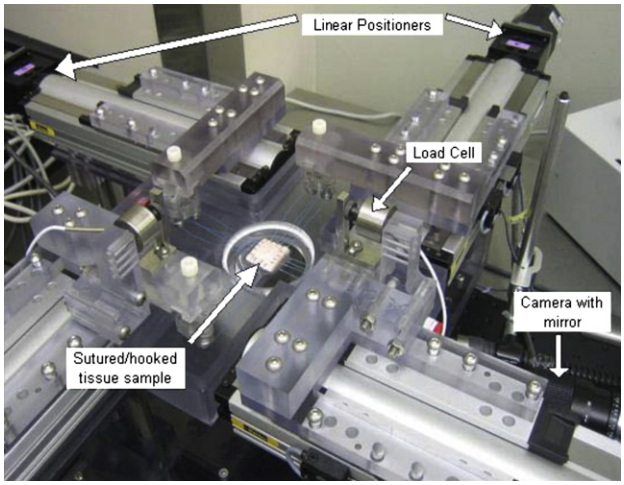

FIG. 1. The biaxial tensile testing device.

Mechanical Testing

The biaxial tensile testing device used in the study has been applied in many other studies of soft tissue mechanics [23–26]. With the device shown in Fig. 1, the tissue can be pulled in two axes with four independent linear positioners. The tissue was hooked on its edges and connected to the linear positioners with sutures as has been done in previous literature [21, 26]. Load cells mounted in the two directions allow the user to control the loading *via* a Labview program (Ver. 6.0, National Instruments, Austin, TX, USA). Initially, a small preload (3 ± 0.050 *g*) was applied in order to straighten the sutures connecting the tissue to the device. Each sample was subjected to a series of eight preconditioning cycles in which the tissue was loaded equally in both directions to a maximum load of 40 g. The actual test of the material was done by applying an equal biaxial load of 200 *g* to the tissue for eight cycles with a load control protocol. During each cycle, the motors applied a load on the tissue starting at the preload state and rising to the maximum load in a 15 s half time. The motors then drop from the maximum load to the preload over the same 15 s half time to complete one cycle. Data used for the analysis was collected from the eighth trial, when the stress-strain curves had become stable. The displacement of four marker dots on the tissue is tracked with a CCD camera at 7–8 Hz in order to measure the strain as a result of the applied loads.

Stress-Strain Calculations

In order to calculate the stresses in the tissue, several assumptions were made. Because the thickness of the samples was much smaller than the length and width dimensions, a plane stress situation is assumed. In addition, the arteries were assumed to be incompressible [28] so the volume of the sample was assumed to be conserved during the loading and unloading process. With these two assumptions, the Cauchy stress (load/deformed area) can be determined using initial tissue dimensions and the load and stretch data from the biaxial testing device:

$$\sigma_1 = \frac{F_1 \lambda_1}{L_{2o} t}, \quad \sigma_2 = \frac{F_2 \lambda_2}{L_{1o} t} \tag{1}$$

In eq 1, σ is the Cauchy stress, F is the applied load, λ is the stretch, L_o is the initial length, and t is the original thickness of the tissue. The subscripts *1*s and *2*s indicate the longitude and circumference directions of the tissue, respectively. In addition, logarithm strain was plotted along with the Cauchy stress in the stress-strain graphs.

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Collagen Assay

The amount of salt soluble, acid soluble, pepsin soluble, and insoluble collagen was determined following the extraction procedures described in detail by Reddy [29]. After separating the volumes of different types of collagen, the samples were analyzed using a Sircol collagen assay kit following manufacture instructions (Biocolor; www.biocolor.co.uk). The Sircol collagen kit uses a quantitative dye-binding method that allowed for the determination of amounts of salt soluble, acid soluble, pepsin soluble, and insoluble collagen. The differences in these types of collagen are minor but they vary in content of high molecular weight cross-links, amino acid sequences, and collagen conformation [30–32]. For the collagen assay, samples were cut into small strips about 2 mm × 1 cm and a wet weight was measured while the tissue samples was fresh. The samples were then submerged in lactated Ringer's solution and placed in the desired storage condition (4°C, –20°C, or –80°C). After the desired storage time (48 h, 1 wk, or 3 wk), the tubes were removed from storage and thawed to room temperature in air for about 1 h.

The strips were then minced and placed in a 2 mL centrifuge tubes with a 1 mL solution of 0.05 M Tris-HCl buffer containing 1.0 M NaCl, 20 mM EDTA, and 1 mM PMSF. This mix was shaken for 24 h at 4°C. Afterwards, the suspension was centrifuged for 15 min at 14,000 rpm, and the supernatant was collected for the Sircol collagen assay to determine the amount of salt soluble collagen. The remaining pellet was resuspended in 0.5 M acetic acid and once again shaken for 24 h at 4°C. The supernatant after this digestion was collected after 15 min of 14,000 rpm centrifugation and used to determine the concentration of acid soluble collagen. The remaining pellet was resuspended in 0.5 M acetic acid containing 1 mg/mL pepsin. This mix was shaken for 24 h at 4°C, and the supernatant, after the same centrifugation as before, was collected as the pepsin soluble collagen. The remaining pellet, after the three soluble collagen extractions, was then diluted with 1 mL of deionized water and heated for 1 h in at 80°C. This process caused the breakdown of the insoluble collagen into soluble collagen so that it could be measured by the Sircol assay kit. Absorbance was measured using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) at a 540 nm wavelength, and was used to determine the collagen content.

Statistical Analysis

Paired t-tests were performed to compare the fresh and stored measurements from each sample to determine if they were statistically significantly different. Paired t-tests were used for the measurements of initial slope, stiff slope, and the stress and strain values of the knee points. A t-test assuming unequal variances was used to compare the collagen contents obtained in the collagen assay. In both analyses, a $P < 0.05$ was used to consider conditions being statistically significant. Results are presented as mean ± SD and in plots error bars are also one SD.

RESULTS

Mechanical Changes

Figure 2 shows a representative set of stress-strain curves. As seen in the plot, the arteries were anisotropic with the circumference direction being stiffer than the longitude direction of the tissue. In order to compare the different tests, a linear region at the beginning section of the stress-strain curve was assumed [33]. The slope of this initial region was called the "initial slope." In order to compare the stiff regions of the stress-strain curves, the linear fit of the curve between 70 and 80 kPa was used because all curves consistently reached this range. This was defined as the "stiff slope." The linearization of the initial and stiff regions was similar to what was done in previous literature [34–36].

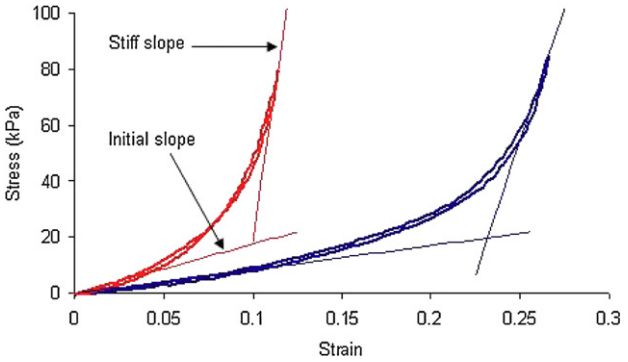

FIG. 2. Representative stress-strain curves showing the determination of initial and stiff slopes.

Figure 3 shows a set of stress-strain curves from both the fresh and stored tissue. The solid lines are from the tests when the sample was fresh, while the dashed lines are from the tests of the same sample after the cold storage. Also shown in Fig. 3 are the "knee points" of the circumference and longitude directions. The knee point strain was located by fitting the stress-strain curve with a fifth order polynomial [37]. After finding the local maximum in the curvature, the corresponding strain at that point was defined as the "knee point strain." The "knee point stress" was then found from the corresponding stress-strain curve.

In Table 1, the initial slopes for the three storage conditions (4°C, –20°C, and –80°C) at three time points (48 h, 1wk, 3 wk) are shown. The L and C refer to the longitude or circumference direction, respectively, throughout this study. The refrigerated samples (4°C storage) showed a significant decrease in the initial slope in both the longitude and circumference directions. For the frozen samples no clear trend was observed and so

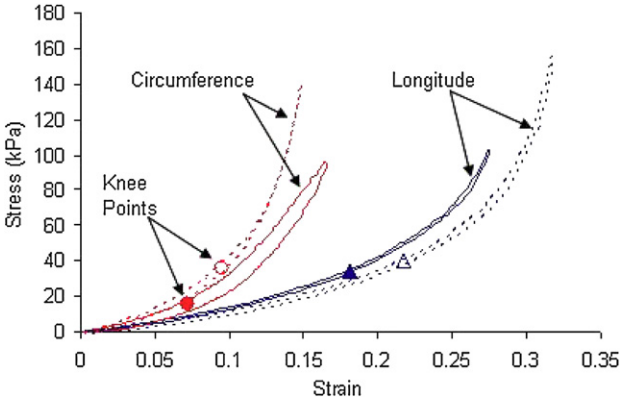

FIG. 3. Representative stress-strain curves from an artery stored at –20°C for 3 wk. Solid lines are from the fresh test, dashed lines are from the testing after storage. The knee points are denoted with circles (circumference direction) and triangles (longitude direction).

TABLE 1

Slopes of Initial Region in Arteries with 4°C, –20°C, and –80°C Storage

Artery group			Fresh slope (kPa)	Stored slope (kPa)	Significance
4°C	48 h	L	149.7 ± 19.7	103.4 ± 13.2	yes
		C	197.1 ± 55.3	171.4 ± 51.0	yes
–20°C	48 h	L	114.8 ± 21.6	85.5 ± 11.4	yes
		C	166.6 ± 22.8	162.9 ± 21.1	no
	1 wk	L	119.5 ± 18.2	94.7 ± 6.8	yes
		C	177.3 ± 43.6	185.7 ± 5.8	no
	3 wk	L	101.0 ± 21.3	91.6 ± 18.7	no
		C	196.9 ± 17.1	180.1 ± 26.8	no
–80°C	48 h	L	132.7 ± 41.1	123.3 ± 14.9	no
		C	188.2 ± 48.0	234.8 ± 29.2	yes
	1 wk	L	137.2 ± 19.0	118.1 ± 6.3	yes
		C	195.0 ± 54.3	219.1 ± 34.0	no
	3 wk	L	135.5 ± 35.1	132.3 ± 29.7	no
		C	200.6 ± 58.5	257.5 ± 50.6	yes

L and C refer to the longitude and circumference directions, respectively.

*$P < 0.05$.

there was no significant change in the initial slopes as a result of freezing storage.

Table 2 shows the stiff slopes for the storage conditions and time points. The refrigerated samples showed a significant increase in the stiff slope in both directions after storage. A significant increase in the stiff slope was also evident in the –20°C storage condition at all time points in both directions. In the –80°C storage condition, there was also a significant increase in stiff slope in the circumference direction at all time points. The longitude direction however only showed a significant change in the stiff slope at the 3 wk time point in –80°C storage. The data in Tables 1 and 2 are summarized in Fig. 4. Each stored condition was normalized to its corresponding fresh condition to show the change in slope after storage.

TABLE 2

Slopes of Stiff region in Arteries with 4°C, –20°C, and –80°C Storage

Artery Group			Fresh slope (kPa)	Stored slope (kPa)	Significance
4°C	48 h	L	925.5 ± 396.4	1019.9 ± 445.1	yes
		C	1199.0 ± 265.8	1617.8 ± 413.8	yes
–20°C	48 h	L	834.0 ± 272.3	1348.9 ± 235.8	yes
		C	1226.7 ± 530.5	2045.0 ± 924.3	yes
	1 wk	L	899.2 ± 215.6	1385.4 ± 291.7	yes
		C	1173.3 ± 353.3	2832.5 ± 884.7	yes
	3 wk	L	800.8 ± 101.6	1265.9 ± 296.5	yes
		C	867.6 ± 296.4	1411.4 ± 387.4	yes
–80°C	48 h	L	902.2 ± 298.1	764.3 ± 220.4	no
		C	976.8 ± 160.7	1466.6 ± 408.9	yes
	1 wk	L	768.6 ± 105.4	738.4 ± 141.2	no
		C	1066.4 ± 233.3	1760.4 ± 927.4	yes
	3 wk	L	758.2 ± 105.2	1013.0 ± 326.3	yes
		C	1350.8 ± 284.4	2277.1 ± 849.3	yes

L and C refer to the longitude and circumference directions, respectively.

*$P < 0.05$.

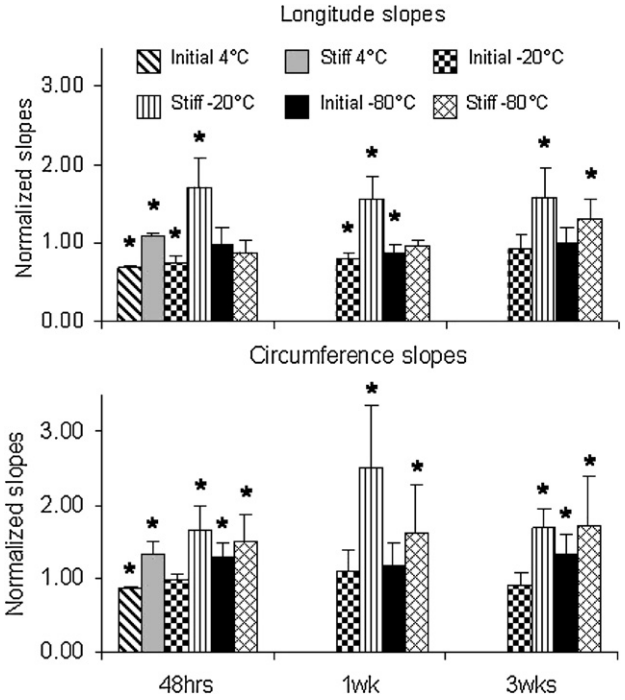

FIG. 4. Normalized initial and stiff slopes after storage at 4°C, –20°C, and –80°C. Each group was normalized to its fresh condition. *$P < 0.05$.

The knee positions of the curves are shown in Fig. 5. The knee strains in the longitude direction increased significantly after storage for all (4°C, –20°C, and –80°C) storage conditions and time points (48 h, 1wk, and 3 wk). There was a trend for an increase in the knee point stress in the longitude direction but the changes were significant in only some of the conditions. In the circumference direction, there was also a significant shift to a higher knee point strain after storage in all conditions except for the 1 and 3 wk conditions at –80°C storage. The circumference direction showed a significant shift to a higher knee stress in all conditions except for 48 h at –20°C.

Biochemical Changes

The biochemical changes in the collagen network were investigated in this study by examining the changes in collagen content and amount of collagen cross-linking. Figure 6 shows the results from the collagen assay. All values are reported as ug of collagen per mg of wet tissue weight. The amounts of salt soluble, acid soluble, pepsin soluble, and insoluble collagen were measured for each storage condition. The amounts of collagen in the stored sample were compared with the corresponding fresh condition values. After 48 h storage, the salt soluble collagen decreased in all storage

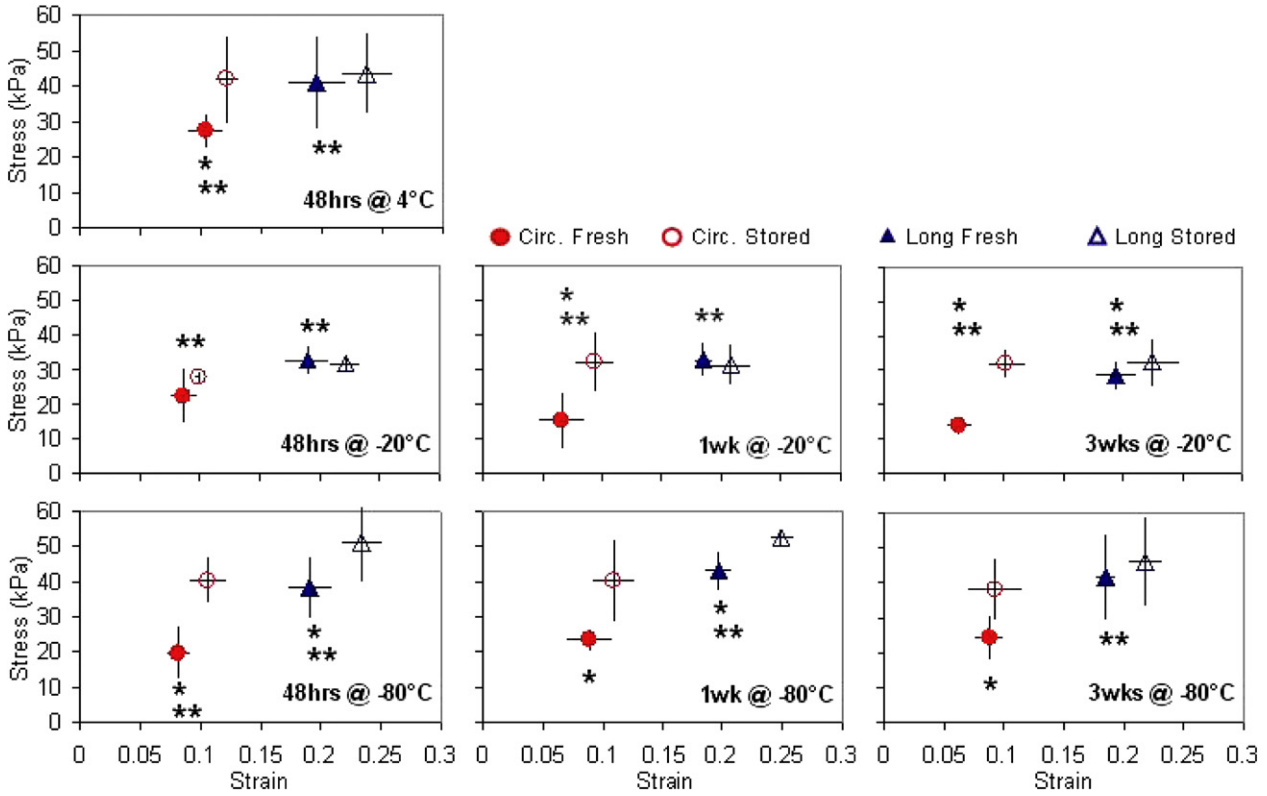

FIG. 5. Comparison of knee points between fresh and stored conditions. The circles and triangles denote the circumference and longitude knee positions, respectively. Filled symbols are from the fresh test. Open symbols are from the tests after the sample has been stored. $*P < 0.05$ for the knee stress. $**P < 0.05$ for the knee strain.

methods. This was expected as the salt soluble collagen is most easily degraded of the three types of soluble collagen. The reason that the salt soluble collagen levels returned to values similar to the fresh samples after 1 and 3 wk storage was not clear. In the samples that were frozen for 1 and 3 wk, there was a significant decrease in the amounts of acid soluble, pepsin soluble, and insoluble collagen.

Figure 7 shows collagen content in the fresh and stored tissue. The soluble collagen was the sum of the salt soluble, acid soluble, and pepsin soluble collagen measurements. The total collagen was calculated by adding the total soluble collagen with the insoluble collagen. The percent of cross-linked collagen is determined by dividing the amount of insoluble collagen by the total amount of collagen and is displayed beneath the pairs of bars [29, 38]. After 48 h storage, there was no significant change in soluble or total amounts of collagen in any storage conditions, although the mean values did show a slight decrease from the fresh condition. However, after 1 and 3 wk of freezing, there were significant decreases in soluble and total collagen in both –20°C and –80°C conditions. The percent of cross-linked collagen in all stored conditions was not significantly different from the fresh condition except for the 1 wk –20°C condition.

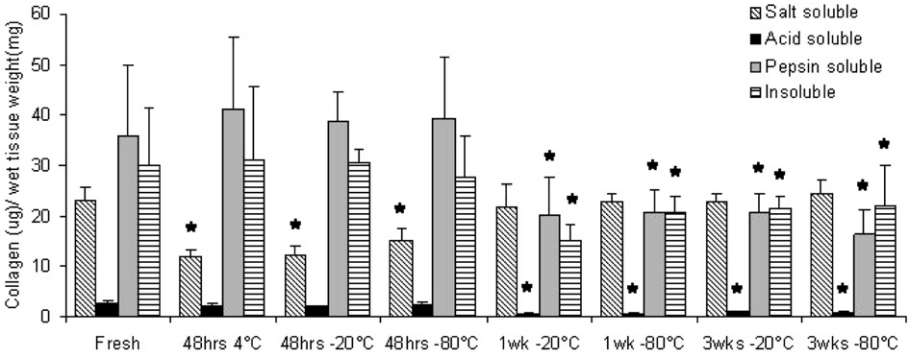

FIG. 6. Results of collagen assay showing the contents of salt soluble, acid soluble, pepsin soluble, and insoluble collagen. All values are reported as ug of collagen per mg of wet tissue weight. $*P < 0.05$.

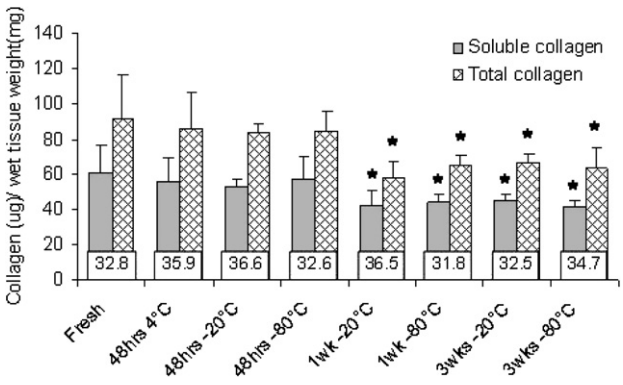

FIG. 7. Results of collagen assay showing the contents of soluble and total collagen. The percentage of cross-linked collagen is displayed in the box. $^*P < 0.05$

DISCUSSION

Similar to our results, previous studies have also reported a decrease in insoluble and total collagen as a result of cold storage [39–41]. We chose to use the Sircol collagen assay kit, which has been demonstrated to successfully quantify the collagen content in biological tissues in previous studies [42–45]. Other studies have used a hydroxyproline assay method but have followed very similar extraction procedures for the soluble and insoluble types of collagen [46–49]. The content of collagen, elastin, and other components of vascular tissue may vary between species [50], but the components of the arteries are similar. Although our results were based on tests with bovine arteries, we expect that the results of studies on the cold storage effects on arteries of other species to be qualitatively similar.

Previous studies have found different results on the effect of temporary cold storage on the mechanical properties of arteries. Using uniaxial tests, Venkatasubramanian *et al.* [19] found that freezing porcine arteries at –20°C significantly increased the Young's modulus of the initial region in the stress-strain curves. Stemper *et al.* [34] investigated the effects of 4°C refrigeration, –20°C freezing, and –80°C freezing storage conditions on porcine arteries. They found a significant decrease in the Young's modulus in the initial region for the refrigerated arteries but the frozen arteries did not show this trend. Also, they showed that fresh and frozen arteries had similar ultimate tensile strength (UTS) but a decrease in UTS was caused by 4°C refrigeration. In another study, increased UTS in collagen-based blood vessel constructs after freezing was reported [51]. The differences in these findings could be due to the different storage procedures. For example, Stemper *et al.* used lactated Ringer's solution and placed arteries in the freezer/refrigerator to reach the desired temperature. In contrast, Venkatasubramanian *et al.* used PBS solutions and radially froze the arteries using a tapered aluminum probe inserted in the arterial rings to achieve radial freezing. In the current study, biaxial tensile testing was used to characterize the mechanical behavior of arteries. This is important to fully understand the multi-axial behavior of anisotropic biological tissue. Moreover, for the first time, changes in the structural component, collagen, is used to explain the mechanical functionality of stored arteries.

Cryopreservation or storage in liquid nitrogen has been clinically used to preserve allografts [4, 12, 13]. Previous studies have shown decellularization of allografts along with cryopreservation with DMSO can improve antibody response and reduce the destruction of the grafts after implantation [6, 7, 9, 52]. Also, studies have suggested that after implantation, cryopreserved grafts may experience less dilatation [2, 53, 54]. Results from earlier studies reported that cryopreserved tissue behaves similar to fresh tissue and has additional benefits of improved antibody response [1, 4, 55]. Although it was beyond the scope of this study to include cryopreservation, the general approach used in this study can be extended to studies on cryopreserved tissue in the future.

Knee Point of Stress-Strain Curves

All the stress-strain curves displayed orthotropic hyperelasticity, which is common in arteries and has been shown in previous literature [56–60]. It is generally accepted that in the initial region, the elastin fibers bear the majority of the load [21, 61, 62]. The knee point is an indication of when the collagen fibers are being recruited to help bear the loading, and so the stress-strain curve transitions from the initial slope to the stiff slope [15, 19]. As the collagen fibers are straightened out and become aligned, there is an increase in stiffness. The stiff region is a result of all the collagen fibers having been recruited and being aligned/pulled upon [21, 42].

The collagen assays showed no significant change in the percent of cross-linked collagen for stored samples (Fig. 7). Shifting of the knee points in the longitude direction to a higher strain after storage (Fig. 5) might correlate with the decrease of total collagen content. With less total collagen content in the tissue after 1 and 3 wk freezing storage, the elastin had to bear the load for a longer period of time before the collagen fibers were recruited, which led to the shift to a higher knee strain. While the total collagen content was not significantly reduced in the 48 h samples, there was a significant decrease in the amount of salt soluble collagen. This would also have led to the similar increase in knee point strain. With the increase in knee point strain and no change in initial slope, it was reasonable that the knee point stress would be higher.

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The shifting of knee point stress and strain in the circumference direction can be explained similarly. However, in some of the stress-strain curves in the circumference direction, the transition from initial to stiff region was more gradual. This caused the curvature to not have a clear local maximum. Similar problems have been reported when trying to find yield point [63, 64]. Thus, the significant increases in knee point strain and stress in the circumference direction may not be as conclusive.

Changes in Initial Slope

The changes in the initial slopes (Table 1, Fig. 4) were similar to those from Stemper *et al.* [34], who found significant decreases in slope when samples were only refrigerated at 4°C but no significant differences in the slopes after freezing. The lack of a change in initial slope in the frozen samples indicated that the properties of the artery were better preserved at freezing temperatures. Through histology studies, Giannini *et al.* [20] observed fragmentation of collagen bundles during the process of freezing and attributed it to ice crystal formation. A possible conclusion from our data is that the formation of ice crystals does not permanently disrupt the elastin network since the stored artery still behaved similar to a fresh artery in the initial region of the stress-strain curve.

Previous studies have shown that there was significant damage to the contraction and relaxation response of endothelial and smooth muscle cells after storage [11, 65]. Histology stains and SEM/TEM scanning have demonstrated that storage over 48 h in 4°C causes partial/complete detachment of and formations of vesicles in the endothelial layer [10, 11, 65, 66]. Another study showed that freezing arteries without excess medium better preserved the cellular junctions and overall structure of the endothelial layer compared to the arteries that were frozen while submerged in medium [67]. The arteries in our study were frozen in lactated Ringer's solution and thus may have experienced destruction in the intima according to previous findings. However, the intima and smooth muscle cells in general are not likely to provide a significant amount of structural support compared with the elastin and collagen network [19, 42]. In future studies, using elastin assays to determine possible changes in elastin concentration and amounts of cross-linking within the elastin network would be useful to understand the lack of change in the initial slope [68].

Changes in Stiff Slopes

The collagen assay showed a significant decrease in the total amount of collagen after freezing conditions for the 1 and 3 wk time points (Fig. 7). It is known that the formation of ice crystals from freezing can disturb the collagen network by splitting and fragmenting collagen bundles [18, 20]. The decrease in the insoluble collagen could be due to the freezing process breaking cross-links and releasing of the now uncross-linked collagen fibers.

Both collagen cross-linking and total collagen content are factors in determining the stiffness of the tissue [15]. Previous studies have found that there was a positive relationship between the amount of cross-linked collagen and tissue stiffness [29, 38, 69]. In addition, some studies have noted that collagen cross-linking maybe a greater determinant of stiffness compared with total amounts of collagen [69–72]. In the present study, the percent of cross-linked collagen was unchanged due to storage (Fig. 7). However, the increases in slope in the stiff region due to storage (Table 2, Fig. 4) were consistent with the results from Venkatasubramanian *et al.* [19]. An increase in diameter of samples after freezing has been previously reported [3, 19, 20]. In our study, we also noticed that the stored samples were slightly larger than the fresh ones particularly in the circumference direction. Thus, it is possible that the collagen fibers in the stored samples were in a more stretched/aligned state that might have led to an increased slope in the stiff region.

The cause for the change in stiff slope might also come from the different procedure for obtaining the slope of the stiff region. In the present study, the stiff slope was obtained by assuming a linear region between stresses of 70 and 80 kPa. However, in a previous study, the tissue was loaded until failure with uniaxial tensile testing and the maximum slope of the stress-strain curve was defined as the stiff slope [29]. The stiff slope determined in this manner corresponds to the point where collagen fibers were fully recruited and were being pulled to failure. In contrast, the stiff slope in our study might not reflect the point where all the collagen fibers were fully stretched.

CONCLUSIONS

To the best of our knowledge, this is the first study that attributes the mechanical properties of aortic tissue to the biochemical changes in the collagen network due to cold storage. The 4°C refrigeration led to a decreased initial slope and an increased stiff slope after 48hrs of storage. The -20°C and -80°C storage conditions did not show changes in the initial slope in most time points but did have an increase in the stiff slope. The knee points of the stress-strain curves were changed as a result of storage. There was a significant

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increase in the knee strain and stress in both the longitude and circumference directions after storage. Collagen assay results show that there was a significant decrease in the soluble and insoluble collagen content but unchanged percentage of cross-linked collagen. Changes of initial slopes, knee points, and stiff slopes due to storage were attributed to possible biochemical and microstructural changes in the artery due to storage. Overall, freezing protocols (either −20°C or −80°C) are suggested over refrigeration (4°C) for maintaining the initial stress-strain behavior of arteries. However, both storage methods (freezing or refrigeration) do result in changes in the mechanical properties due to structural and biochemical changes in the tissue. Future studies would involve elastin assays as well as microscopy techniques for further understanding the biochemical and structural changes due to storage. In addition, adding liquid nitrogen and cryopreservation with DMSO storage conditions could be useful to examine techniques currently used in allograft storage.

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