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Research paper

Impact of cold storage protocols on passive arterial wall mechanical behavior

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ABSTRACT

Mechanical characterization of arterial tissue is essential for understanding vascular function, pathologies, and for developing computational models and vascular devices. However, tissue storage prior to ex vivo testing can alter mechanical behavior, complicating data interpretation and comparability. This study systematically evaluated how commonly used cold storage protocols affect the passive viscoelastic behavior of the arterial wall.

Specimens (n=177) from porcine descending aortas (n=12) underwent biaxial mechanical testing either fresh or after cold storage. Storage protocols (n=6) varied in temperature, freezing rate, and cryoprotectant use. Stored strain energy density during loading and strain energy density difference between loading and unloading cycles (loss factor) quantified elastic and viscous behavior, respectively. Linear mixed-effects models accounted for variability among animals and anatomical locations.

Viscous behavior was largely preserved, whereas elastic behavior was significantly affected by cold storage. Refrigeration decreased strain energy density, while snap freezing and cryoprotectant use increased it, with more pronounced effects under circumferential loading. Slow freezing in phosphate-buffered saline to -80°C best preserved the passive mechanical behavior. Variability among animals and anatomical locations dominated variance in elastic (55%–65%) and viscous (~65%) behavior, exceeding storage effects (15%–20% and <5%, respectively).

Although limited to healthy porcine tissue and without direct assessment of the underlying mechanisms, these findings demonstrate that cold storage alters passive arterial mechanics, particularly elasticity, while variability among animals and anatomical locations is a critical source of variation. This study provides guidance for selecting appropriate storage protocols pre-mechanical testing and highlights the importance of variability among test subjects and anatomical locations.

1. Introduction

Mechanical characterization of arterial tissue is fundamental to understanding vascular physiology, disease progression, and the development of computational and experimental models in cardiovascular biomechanics. Accurate quantification of arterial mechanical properties is essential for applications ranging from patient-specific modeling to the design and evaluation of medical devices. However, the reliability and comparability of such measurements remain challenging, largely due to the inherent variability of biological tissues and differences in experimental protocols. A community-driven initiative *C⁴Bio* (Famaey et al., 2026) has further highlighted that even well-controlled testing protocols can introduce substantial variability in measured mechanical parameters, raising important questions about the relative contribution of experimental factors to the observed mechanical response.

One important source of experimental variability arises from tissue handling and storage prior to mechanical testing. Because immediate testing of freshly excised tissue is often impractical, a range of storage protocols is commonly employed, including short-term refrigeration and freezing with or without cryoprotective agents. While these approaches aim to preserve tissue integrity, they may still induce structural and biochemical alterations, and hence changes in tissue mechanical behavior. The existing literature on the effect of cold storage on arterial wall material behavior is unfortunately inconclusive. For example, O'Leary et al. (2014) reported negligible changes in the mechanical response of porcine thoracic aortas after storage up to 12 months at -20°C , while Chow and Zhang (2011) observed a significant increase in stiffness in human aortic segments after comparable storage durations. Similarly, Stemper et al. (2007) found that frozen human arteries retained their stress-strain behavior, while Hemmasizadeh et al.

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(2012) documented altered viscoelastic properties in porcine aortas subjected to comparable storage protocols. Yin et al. (2012) and Ghosn et al. (2011) reported contradictory results with respect to low-strain mechanical behavior, where one study noted an increase in stiffness and the other did not. These discrepancies are compounded by differences in experimental design, tissue type, and sample size, making it difficult to establish generalizable conclusions.

At the same time, arterial tissues exhibit substantial biological heterogeneity, both between subjects and within a single vessel depending on anatomical location. This variability can obscure the effects of storage, particularly when not explicitly accounted for in the analysis. As a result, it remains unclear not only whether storage protocols induce significant changes in mechanical behavior, but also whether these changes are meaningful relative to the underlying inter- and intra-subject variability. Moreover, arterial mechanics are inherently anisotropic and viscoelastic, with distinct responses in circumferential and longitudinal directions. Storage-induced alterations may therefore depend on loading direction and mechanical metric, yet these aspects are not consistently addressed in the literature, further complicating interpretation.

The aim of this study was to systematically evaluate the impact of commonly used storage protocols on arterial mechanics, while explicitly accounting for variability among subjects and anatomical locations. In this context, the study focuses exclusively on the passive mechanical behavior of the arterial wall. Porcine descending aorta specimens were subjected to multidirectional mechanical testing following fresh, refrigerated, and multiple freezing protocols, including variations in freezing rate and cryoprotectant use. Linear mixed-effects models were employed to quantify storage-induced effects by correcting the results for variability among animals and anatomical locations. By evaluating both elastic and viscous properties, the influence of cold storage methods on passive arterial wall behavior was assessed, and the storage protocols that reliably maintain mechanical integrity for arterial testing were identified.

2. Materials and methods

2.1. Tissue collection and specimen preparation

Descending thoracic aortas were excised from 12 adult pigs at a local slaughterhouse and transported to Erasmus Medical Center in phosphate-buffered saline (PBS) at +4 °C. Perivascular fat and connective tissue, including the loose, outermost portion of the adventitia, were carefully removed. This ensured reliable thickness measurements obtained by high-resolution ultrasound in a fluid bath (see Section 2.3), as it minimized ambiguity at the boundary caused by loosely organized fibers that tend to swell in a fluid. Next, square specimens measuring 15 × 15 mm² were excised at predefined locations along the aorta, yielding a total of 191 test specimens. These locations were determined using a template with intercostal bifurcations as landmarks, as shown in Fig. 1(a).

2.2. Storage protocols

All specimens were randomly assigned to one of seven storage protocols (six experimental groups and one control group), resulting in approximately 27 specimens per group. The selected protocols were informed by a comprehensive literature review that identified the most commonly employed cold storage strategies prior to mechanical testing of arterial tissue.

To capture the range of approaches typically used in laboratories, the study included fresh specimens (control group), short-term refrigeration, and slow and snap freezing with or without the use of a cryoprotective agent. These choices allowed systematic evaluation of both freezing rate and cryoprotectant use. To limit the total number of conditions, commonly reported parameters were fixed: specimens

stored in a freezer were kept at −80 °C, PBS was used as the storage medium for wet storage conditions, and DMSO was employed as a cryoprotective agent. A DMSO concentration of 10% (v/v) was used, as this concentration is commonly reported in studies evaluating the effects of storage protocols on tissue mechanical properties (Langerak et al., 2001; van Doormaal et al., 2014; Bia et al., 2006; Blondel et al., 2000; Virues Delgadillo et al., 2010) and is widely regarded as providing a favorable balance between cryoprotective efficacy and cytotoxicity in cryopreservation applications. Previous studies have reported no systematic differences in arterial wall mechanical properties attributable to the choice of storage medium (e.g., Ringer's solution, PBS, or RPMI), demonstrated superior performance of DMSO compared to glycerol (van Doormaal et al., 2014), and shown improved preservation with storage at −80 °C compared to −20 °C (Hemmasizadeh et al., 2012).

To enable broad accessibility and reproducibility across laboratories in the future, storage protocols requiring specialized equipment (e.g., rate-controlled freezing) were excluded. Previous work has shown no significant difference between slow freezing and rate-controlled freezing on the structure or mechanics of arterial tissue (Kostelnik et al., 2024). In addition to the commonly reported protocols, an additional storage method using pre-cooled isopentane, which has been shown to be optimal for preserving tissue structure (Hoeier et al., 2025), was included. Its effects on mechanical properties have not been previously evaluated; therefore, it was incorporated to determine whether the preservation advantages for tissue structure would also extend to mechanical behavior. All frozen specimens were stored for one month. Previous studies have demonstrated that, while freezing itself may influence tissue mechanical properties, extending storage up to at least three months does not produce further measurable changes (O'Leary et al., 2014; Kovács et al., 2025; Lőrinczy and Fazekas, 2022; Lőrinczy et al., 2023).

In the end, the following seven storage protocols were investigated:

1. **Fresh (Control):** Specimens were tested immediately on the day of tissue collection (no storage).
2. **Refrigerated (RFG):** Specimens were placed in a plastic vial with PBS and stored at +4 °C for 1 week.
3. **Slow-Frozen in PBS (F_{PBS}):** Specimens were placed in a plastic vial with PBS and stored in a freezer at −80 °C for 1 month.
4. **Slow-Frozen in PBS with cryoprotectant (F_{DMSO}):** Specimens were placed in a plastic vial containing a solution of PBS and 10% DMSO. The vials were stored in a freezer at −80 °C for 1 month.
5. **Snap-Frozen without medium (S_0):** Specimens were placed in a plastic vial without medium and snap-frozen by immersion of the vial in liquid nitrogen. The vials were then stored in a freezer at −80 °C for 1 month.
6. **Snap-Frozen with cryoprotectant (S_{DMSO}):** Specimens were placed in a plastic vial containing a solution of PBS and 10% DMSO and snap-frozen by immersing the vial in liquid nitrogen. The vials were then stored in a freezer at −80 °C for 1 month.
7. **Snap-Frozen in isopentane (S_{IP}):** Specimens were submerged directly in isopentane cooled to −80 °C and placed in an aluminum container without medium. The containers were then stored in a liquid nitrogen tank at −196 °C for 1 month.

Within 12 h after excision of the aortas, specimens were either mechanically tested (control group) or stored according to the designated protocol. The mean excision-to-storage time was 8.01 ± 2.19 h, and a one-way ANOVA confirmed that there were no statistically significant differences in delay times between groups ($p = 0.965$; Supplementary Table S1). Before mechanical testing, slow-frozen and snap-frozen specimens were thawed at +4 °C. Samples stored in 10% DMSO were then transferred to 5% DMSO for 5 min and subsequently rinsed in PBS for 5 min, both at room temperature, to wash out remaining DMSO and minimize osmotic shock before mechanical testing.

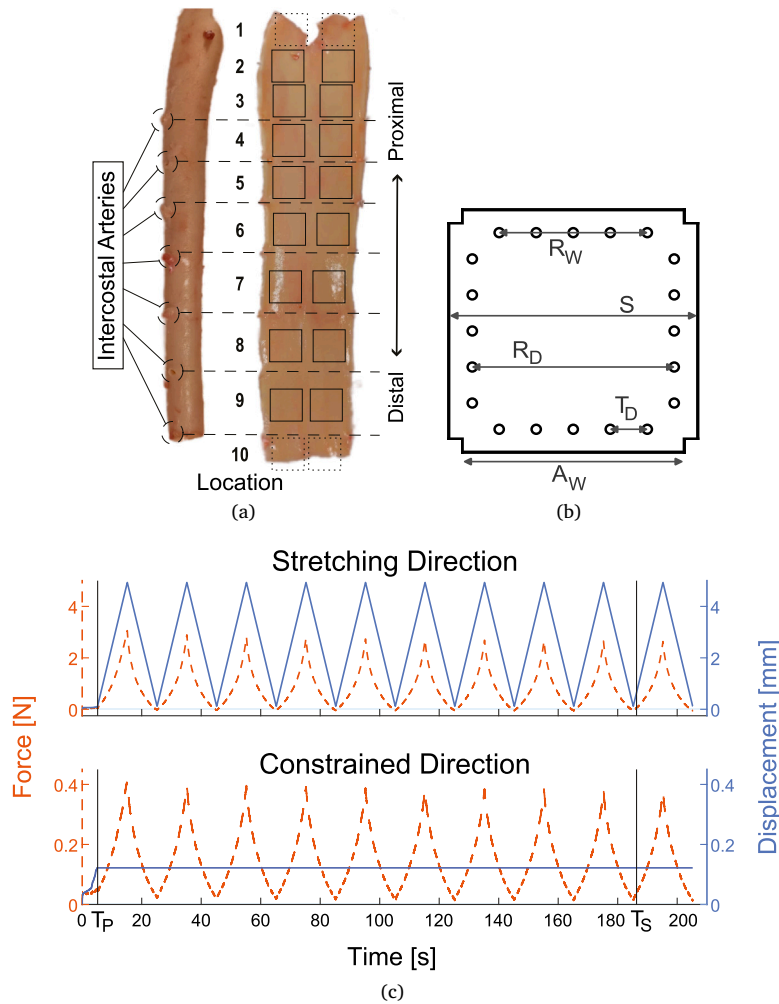


Fig. 1. (a) Specimen location numbering based on the anatomical position of intercostal artery branches, with increasing numbers from proximal to distal side. A representative example of excised specimens is shown on a longitudinally opened artery (right). Specimens could not be obtained from some locations, as indicated by dashed boxes, due to insufficient artery length or irregular end shape. (b) Top view of geometric parameters of the test specimen and rake configuration. The initial square specimen edge length is denoted by $S = 15$ mm. The total rake width R_W , apron width A_W , and the initial rake separation distance R_D are 8.8 mm, 13 mm, and 12 mm, respectively. The spacing between individual rake tines is $T_D = 2.2$ mm and the tine diameter is $305 \mu\text{m}$. (c) Displacement-driven mechanical testing protocol showing force and displacement data over time in the stretching direction and the constrained direction for a representative test. Time points T_P and T_S correspond to the moments when the preload force of 50 mN was reached. T_P marks the onset of the preconditioning protocol and T_S the start of the final loading cycle used for further analysis.

2.3. Mechanical testing

Biaxial testing was selected to characterize the mechanical response of the tissue specimens in both circumferential and longitudinal directions. To avoid desiccation, the specimens were kept hydrated in PBS at room temperature between preparation and mechanical testing. To reduce the apron effect, which influences biaxial mechanics by increasing apparent stiffness (Di Leonardo et al., 2024), $1 \times 1 \text{ mm}^2$ squares were removed from the corners using a scalpel (Fig. 1(b)). A random speckle pattern was applied by spraying the luminal side of the specimen with fluorescent dye using an airbrush. The specimens were mounted onto a biaxial testing device (Biotester 5000, CellScale, Canada) using rakes (Fig. 1(b)) consisting of five tines, each with a diameter of $305 \mu\text{m}$, a puncture depth of 2.4 mm, a total rake width of 8.8 mm, and an initial rake-to-rake distance of 12 mm. The specimens were submerged in PBS at 37°C . In this configuration, the thickness of the specimens was measured through B-mode ultrasound (VEVO 3100, FUJIFILM VisualSonics, Canada) at $100 \mu\text{m}$ intervals using a linear transducer (MX550) with a center frequency of 40 MHz and an axial resolution of $40 \mu\text{m}$.

Each specimen underwent two consecutive loading protocols. First, a circumferential stretching protocol was applied, with engineering strain up to 40%, while the longitudinal displacement was constrained by maintaining the preload dimension. No additional stretch was applied in the constrained direction in order to preserve a simple and reproducible testing protocol. Subsequently, an analogous longitudinal stretching protocol was performed, with circumferential deformation constrained, to obtain comparable data under consistent boundary conditions.

The circumferential stretching protocol was designed to span and moderately exceed physiological aortic stretch levels, enabling characterization of both the quasi-physiological response and the nonlinear stiffening behavior at higher strains. Although this configuration does not fully reproduce the in vivo loading state of the aorta, which includes axial pre-stretch on the order of approximately 10%–20% engineering strain, it preserves mechanical coupling between directions while maintaining a simple and reproducible testing framework suitable for comparative analysis.

The loading protocols were displacement-driven at a strain rate of 4%/s (Fig. 1(c)). At the start of each protocol, a preload of 50 mN

was applied in both directions, followed by 10 loading cycles up to 40% engineering strain in the primary loading direction. Mechanical parameters were extracted from the final, 10th cycle. Stabilization of preconditioning cycles was evaluated based on cycle-to-cycle changes in the force response, with representative data provided in the Supplementary Materials. Force and displacement data were recorded at 100 Hz using a transducer with a maximum load capacity of 10 N and an accuracy of 20 mN (0.2% of full-scale load). Optical images were acquired at 2.5 Hz with a high-resolution camera system (Imager CS1-5, LaVision GmbH, Germany) equipped with a BP600-40 orange band-pass filter, while specimens were illuminated with blue light at 445 nm (LED 35 Illumination Unit, LaVision GmbH, Germany).

2.4. Data processing

2.4.1. Thickness

Manual annotations of thickness measurements were made using Vevo LAB software (FUJIFILM VisualSonics, Canada). For each specimen, measurements were taken at nine locations arranged in a 3×3 grid spanning the central $9 \times 9 \text{ mm}^2$ region of the specimen. Measurement points were positioned sufficiently far from the tines to avoid artifacts arising from acoustic reverberations caused by reflections off the metal tine surfaces. The nine thickness values were averaged to yield a single representative thickness per specimen.

2.4.2. Deformation

Engineering strain in both circumferential and longitudinal directions was calculated using digital image correlation (DIC). DIC computations were performed using DaVis software (LaVision GmbH, Germany). A subset size of 101 pixels and a step size of 66 pixels were used, optimized for the acquired imaging resolution and speckle quality. Strain measurements were averaged over the central 20% of the specimen surface area. To account for temporal mismatches between the start and end points of the loading cycles and the image acquisition, strain values were interpolated and up-sampled to 500 Hz to align with force recordings. The zero-strain state in the specimens was considered to correspond to the time point at which the preload of 50 mN was reached during the final loading cycle (Fig. 1(c)).

From these engineering strains, the principal stretches in circumferential, λ_C , and longitudinal, λ_L , directions were computed as

$$\lambda_C = 1 + \varepsilon_{CC}, \quad \lambda_L = 1 + \varepsilon_{LL} \quad (1)$$

where ε_{CC} and ε_{LL} denote the measured engineering strains in the circumferential and longitudinal directions, respectively.

2.4.3. Stress

The first Piola–Kirchhoff (PK1) stress tensor $\mathbf{P}$ was constructed assuming plane stress:

$$\mathbf{P} = \frac{1}{W \cdot T} \begin{bmatrix} F_C & 0 & 0 \\ 0 & F_L & 0 \\ 0 & 0 & 0 \end{bmatrix} = \begin{bmatrix} P_{CC} & 0 & 0 \\ 0 & P_{LL} & 0 \\ 0 & 0 & 0 \end{bmatrix}. \quad (2)$$

Here, F_i is the measured force in direction i (where $i = C$ for circumferential and $i = L$ for longitudinal), while W and T are the width between the rakes (equal to 12 mm) and average specimen thickness at the start of the test, respectively. The raw force data were up-sampled to 500 Hz, and measurement noise was smoothed out using a forward–backward moving average filter with a window size of 40. Hereafter, all stresses are reported as PK1 stresses.

2.5. Mechanical parameters

Strain energy density Ψ is calculated by

$$\Psi = \int \mathbf{P} : d\mathbf{F} \quad (3)$$

where $\mathbf{P}$ is the PK1 stress tensor, and $\mathbf{F}$ is the deformation gradient tensor, constructed by assuming incompressibility:

$$\mathbf{F} = \begin{bmatrix} \lambda_C & 0 & 0 \\ 0 & \lambda_L & 0 \\ 0 & 0 & 1/(\lambda_C \lambda_L) \end{bmatrix} \quad (4)$$

Using Eqs. (2)–(4), the strain energy density formulation reduces to

$$\Psi = \int P_{CC}(\lambda_C) d\lambda_C + \int P_{LL}(\lambda_L) d\lambda_L. \quad (5)$$

For each specimen, Ψ was calculated for each loading protocol, denoted by i (where $i = \text{Circ}$ for circumferential and $i = \text{Long}$ for longitudinal stretching), at a stretch of $\lambda = 1.1, 1.2$ and 1.3 . Strain energy densities for the loading and unloading cycles are denoted as Ψ_λ^i and $\Psi_\lambda^{i,U}$, respectively, where Ψ_λ^i is calculated using the loading curve and $\Psi_\lambda^{i,U}$ using the unloading curve. The strain energy density represents the mechanical work stored in the tissue per unit reference volume during deformation. Numerical integration was performed using the trapezoidal rule. The mathematical derivation and implementation details are provided in the Supplementary Materials.

To quantify viscoelastic losses, the loss factor for each loading protocol i , denoted by LF_i , was computed as the relative difference in strain energy density between the loading and unloading curves up to the maximum applied stretch $\lambda_{\max}$:

$$LF_i = \frac{\Psi_{\lambda_{\max}}^i - \Psi_{\lambda_{\max}}^{i,U}}{\Psi_{\lambda_{\max}}^i} \quad (6)$$

The loss factor therefore represents the fraction of mechanical energy dissipated during a loading–unloading cycle, reflecting the viscous behavior of the tissue.

2.6. Statistical analysis

All statistical analyses were conducted in R Statistical Software (v4.3.1; R Core Team, 2023). Linear mixed-effects models were fitted using the *lme4* package (v1.1.37; Bates et al. 2015) to account for the repeated-measures design, with storage protocol as a fixed effect and animal identity and anatomical location as random effects. Random-effects structures were selected based on the lowest Bayesian Information Criterion (BIC). Sensitivity analyses were conducted to assess the impact of data imbalance in the random-effects structure. Model assumptions were evaluated via residual diagnostics.

For the LF_{Circ} parameter, right-skewness and heteroscedasticity remained after log transformation; therefore, this parameter was analyzed using a mixed-effects model fitted with the *nlme* package (v3.1.168; Pinheiro et al. 2025), allowing group-specific variance structures. Pairwise comparisons of estimated marginal means were conducted using the *emmeans* package (v1.11.1; Lenth 2025) with Satterthwaite-adjusted t-tests and Tukey correction for multiple comparisons. Statistical significance was set at $\alpha = 0.05$. Model summaries, sensitivity analyses, diagnostic plots, estimated marginal means and pairwise comparisons are provided in Supplementary Materials.

To further characterize the sources of variability within the measured mechanical parameters, variance components for the random effects and residual error were extracted from the mixed-effects models. In addition, the variance explained by the fixed effects was approximated from the fitted values. Total variance was defined as the sum of the random-effect variance components, residual variance, and the variance explained by the fixed effects. The proportional contribution of each component was expressed as a percentage of total variance to quantify the relative influence of storage protocol, inter-animal variability, anatomical location, and unexplained residual variance, following a variance-partitioning approach analogous to marginal and conditional R^2 for mixed-effects models (Nakagawa and Schielzeth, 2013).

To put storage-related effects in relation to biological variability, a biological standard deviation was computed from the variance components of the mixed-effects model as:

$$\sigma_{bio} = \sqrt{\sigma_{animal}^2 + \sigma_{location}^2} \quad (7)$$

where σ_{animal}^2 and $\sigma_{location}^2$ represent the estimated random-effect variances for animal identity and anatomical location, respectively. This quantity was used for post hoc scaling of estimated storage effects.

3. Results

3.1. Stress–stretch response

From 12 descending thoracic porcine aortas, 191 test specimens were excised. Of these, 14 specimens were not included in the stress–stretch evaluation: five due to rake failure (unhooking or slippage during testing), six due to low average thickness (<1.35 mm) associated with pronounced preload-induced stretching and occasional failure to reach the target 30% engineering strain, and three due to highly inhomogeneous strain fields (coefficient of variation >0.1 at the end of loading). The thickness exclusion threshold of 1.35 mm corresponded to the lower bound of a one-sided 95% confidence interval of the observed thickness distribution (mean = 1.645 × SD).

A detailed overview of sample exclusion is provided in the Supplementary Materials. Consequently, 177 specimens were included in the mechanical analysis.

The stress–stretch curves recorded during the last stretching cycle are shown in Fig. 2. Control group specimens exhibited the expected nonlinear, strain–stiffening response under both circumferential and longitudinal stretching. Circumferential normal stresses were greater than the longitudinal ones (20 vs. 14 kPa at $\lambda = 1.1$; 43 vs. 30 kPa at $\lambda = 1.2$; 73 vs. 48 kPa at $\lambda = 1.3$, on average). Nonlinear, strain–stiffening behavior and a stiffer response under circumferential stretching than under longitudinal stretching were also observed across all six storage groups.

Due to the displacement constraint in the transverse direction during biaxial testing, non-zero normal stresses were present in that direction. These stresses were substantially lower than those in the stretching direction. For each sample, the loading and unloading stress–stretch curves recorded during the last stretching cycle in both directions are provided in the Supplementary Materials.

3.2. Effect of storage protocols

The group average stress–stretch response of the control group is plotted in each storage group panel in Fig. 2. This group average curve was obtained by resampling stretch and PK1 stress data for each specimen to 100 evenly spaced points over its individual stretch range, and independently averaging stretch and PK1 stress values at each resampling point. However, it is important to note that a direct comparison of group averages may be misleading since the effect of the storage protocol cannot be isolated from the effect of biological variability, as the test specimens were not independent (specimens from the same animal and/or same anatomical location).

Using mixed-effects statistical models, the mechanical responses of the specimens were adjusted for variability among animals and anatomical locations to compare the effect of the storage protocols. The strain energy density measures (Ψ) and the loss factor (LF) for each storage protocol were chosen as mechanical parameters of interest to describe the viscoelastic characteristics of the tissue. The results of these mechanical parameters per test group are shown in Fig. 3. A summary of the raw and adjusted mechanical data, including pairwise comparisons, is provided in the Supplementary Materials.

For all test groups, strain energy density stored in the specimens increased with further stretching, and the values during the circumferential stretching were greater than those during the longitudinal stretching. For the control group, strain energy density averaged 1.08 kPa

(95% CI: 0.97–1.19 kPa), 4.28 kPa (3.87–4.70 kPa), and 10.19 kPa (9.18–11.20 kPa) at $\lambda = 1.1$, 1.2 and 1.3, respectively. The corresponding values during the longitudinal stretching protocol were 0.81 kPa (0.74–0.88 kPa), 2.99 kPa (2.74–3.24 kPa), and 6.86 kPa (6.28–7.44 kPa).

The refrigeration (*RFG*) group consistently demonstrated statistically significantly lower strain energy density compared to the control group, for both stretching protocols and at all stretch levels. All slow-frozen and snap-frozen groups, except the slow-frozen in PBS (F_{PBS}) group, showed higher strain energy density under circumferential stretching than the control group. These differences were statistically significant for all stretch levels for the cryoprotectant groups (F_{DMSO} , S_{DMSO}) and snap-frozen specimens without medium (S_0), whereas they were so for the snap-frozen in isopentane (S_{IP}) group from stretch values of 1.2 onward. Moreover, the slow-frozen group with cryoprotectant (F_{DMSO}) exhibited a significantly higher strain energy density compared to the slow-frozen group without cryoprotectant (F_{PBS}).

Under longitudinal stretching, the qualitative trends across storage protocols closely matched those observed under circumferential stretching. The refrigerated group (*RFG*) was the only protocol that differed statistically significant from the control group across all stretch levels. Among the snap-freezing protocols, all groups showed increased Ψ^{Long} relative to the control group, but only S_0 reached statistical significance at $\lambda = 1.3$ ($p = 0.042$). The other snap-freezing groups followed the same directional trend, with S_{DMSO} and S_{IP} showing suggestive but non-significant increases ($p = 0.149$ and $p = 0.145$, respectively). In contrast, the slow-freezing protocols showed less pronounced effects, with both F_{PBS} and F_{DMSO} exhibiting smaller, non-significant increases in Ψ^{Long} ($p = 0.449$ and $p = 0.285$, respectively). Similar trends were observed at $\lambda = 1.1$ and $\lambda = 1.2$, with all storage groups again showing increased Ψ^{Long} relative to the control group. However, S_0 did not differ significantly from the control group at these lower stretch levels ($p = 0.175$ at $\lambda = 1.1$; $p = 0.138$ at $\lambda = 1.2$), despite maintaining the same direction of effect as at $\lambda = 1.3$.

Viscous behavior in the specimens, manifested as energy dissipation during cyclic loading, was quantified using the loss factor (LF). LF was comparable between the circumferential and longitudinal stretching protocols in the control group, with mean values of 3.51% (95% CI: 2.78–4.42%) vs. 3.44% (95% CI: 2.99–3.88%), respectively. The *RFG* group exhibited LF values slightly lower, and F_{PBS} slightly higher than the control group in both stretching directions. Although neither group differed significantly from the control group, pairwise comparisons revealed a statistically significant difference between *RFG* and F_{PBS} for both LF_{Circ} and LF_{Long} ($p < 0.001$, $p < 0.003$, respectively). The other frozen and snap-frozen groups demonstrated values comparable to the control group. The cryoprotectant groups (F_{DMSO} , S_{DMSO}) showed lower mean LF values than their respective non-cryoprotected counterparts (F_{PBS} , S_0), but these differences were not statistically significant.

3.3. Variance quantification

Using mixed-effects models, the contributions of storage protocol, animal identity, and anatomical location to variability in mechanical parameters were quantified (Fig. 4). Animal identity and anatomical location combined accounted for roughly 60% of the total variance observed in the strain energy density, regardless of the loading direction and stretch level (i.e., Ψ_{λ}^{Circ} and Ψ_{λ}^{Long} for $\lambda = 1.1$, 1.2 and 1.3). Under circumferential stretching, anatomical location was more important than animal identity, while under longitudinal stretching, it was the opposite. Notably, with increased stretch, the contribution of animal identity increased while that of the anatomical location decreased for both circumferential and longitudinal directions. The choice of the storage protocol consistently explained 15%–20% of the variance. Approximately 20%–30% of the variance could not be explained by any

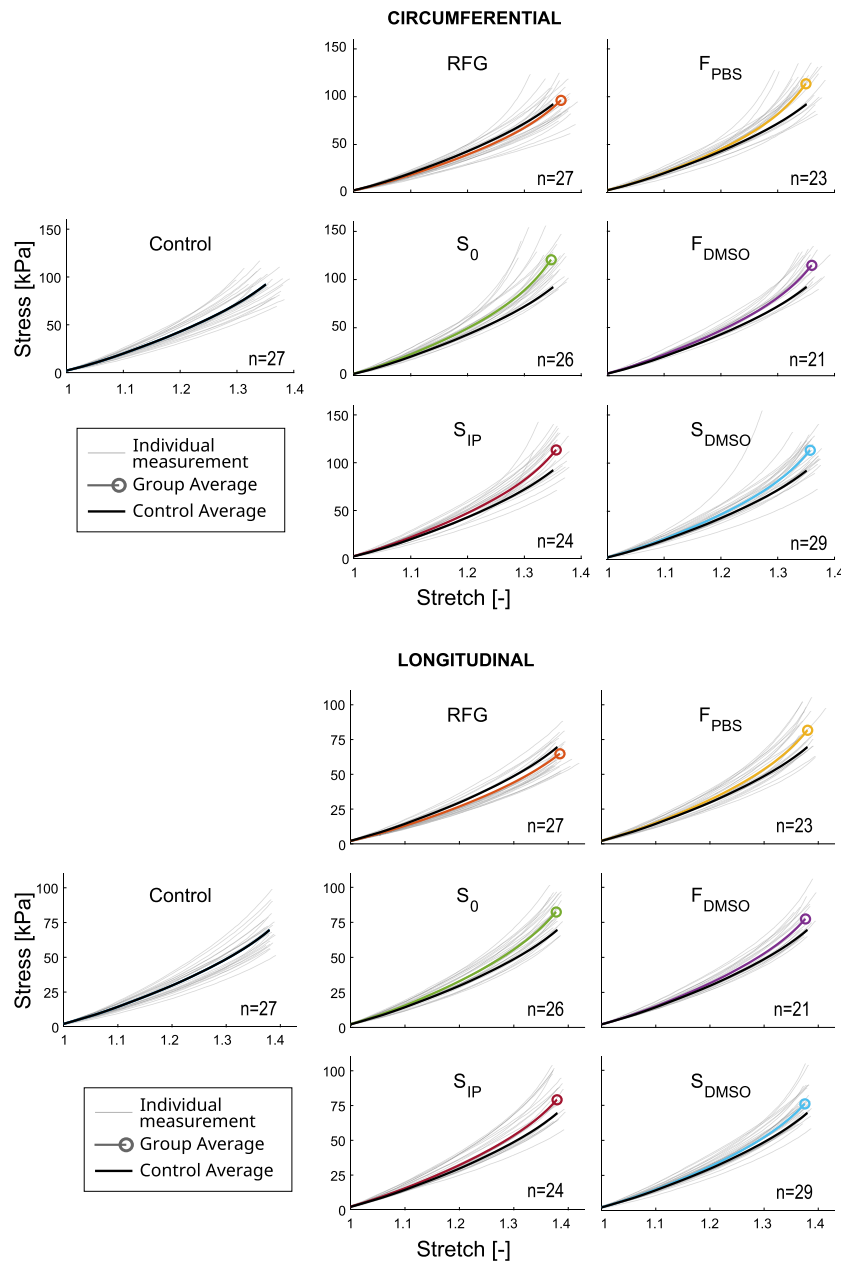


Fig. 2. First Piola–Kirchhoff stress–stretch curves for different storage groups under circumferential (top) and longitudinal (bottom) stretching protocols. The visualized stress and stretch correspond to the main loading direction under each protocol. Individual specimens are shown together with the group average, alongside the control group average. The number of specimens per group (n) is indicated in the bottom right corner.

of these three factors (animal identity, anatomical location, or storage protocol).

For the loss factor, animal identity accounted for a substantial part of the variance (49% in the longitudinal direction and 67% in the circumferential direction). The influence of storage protocol had a negligible contribution to the variance observed in this parameter (2% under circumferential stretching and 4% under longitudinal stretching). The anatomical location accounted for 15% of the variance under longitudinal stretching, while it had a negligible contribution to the total variance under circumferential stretching.

To place storage effects in the context of biological variability, changes in mechanical parameters for each storage protocol were normalized by the biological standard deviation (σ_{bio}), estimated from variance components associated with animal identity and anatomical location (Supplementary Tables S32–S34). For strain energy density, the largest storage effect (*RFG* vs. Control) was comparable to σ_{bio}

($0.8\text{--}1.0 \times \sigma_{bio}$), whereas other effects were generally smaller ($0.1\text{--}0.7 \times \sigma_{bio}$). For the loss factor, normalized storage effects were consistently low ($\leq 0.4 \times \sigma_{bio}$) for all storage groups.

4. Discussion

We observed in our study a clear stretch-stiffening response across all storage groups. Moreover, circumferential stresses exceeded longitudinal stresses over the tested stretch range. These findings confirm the well-known nonlinear and anisotropic passive behavior of the arterial wall. Building on this anisotropic response, cold storage was found to differentially affect the arterial wall mechanics, with a more pronounced impact in the circumferential direction than in the longitudinal direction. Importantly, the width of the confidence intervals for the estimated storage-induced changes in mechanical parameters was similar for both loading directions. This indicates that

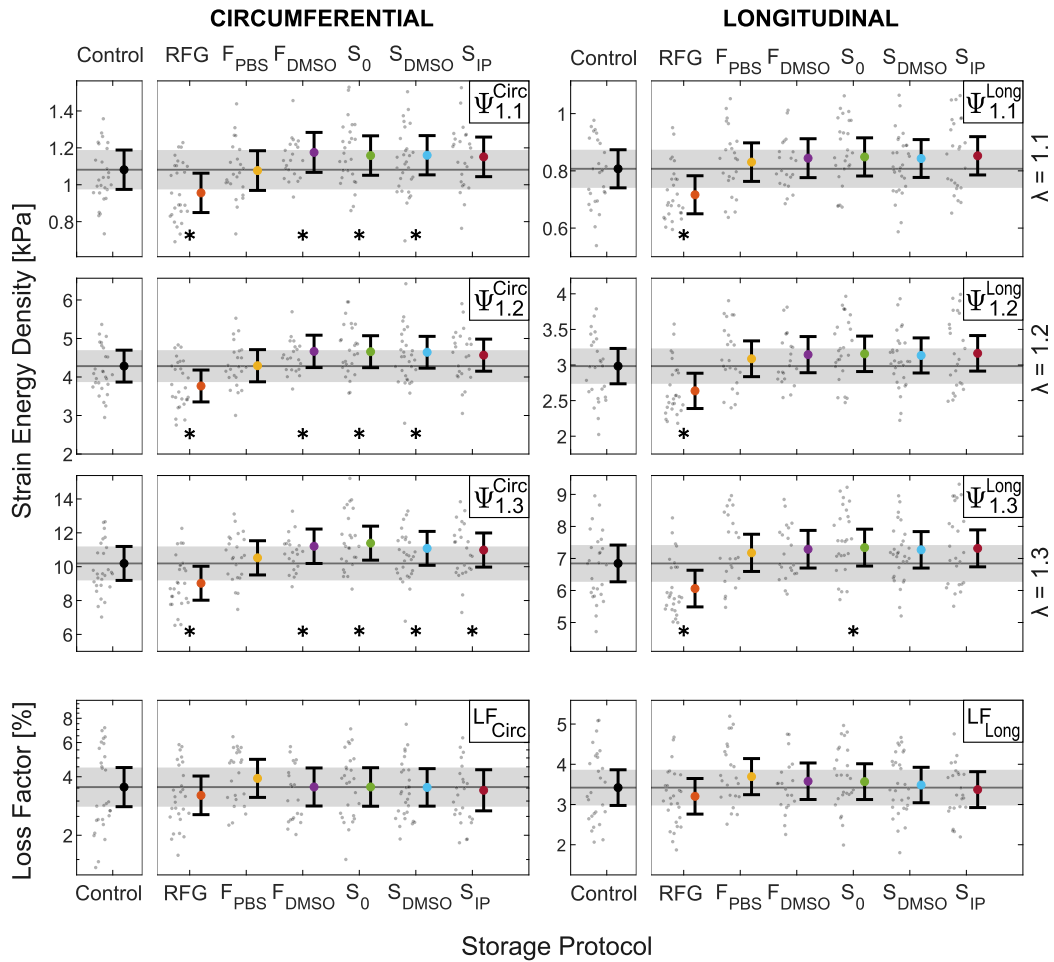


Fig. 3. Strain energy density (top) at $\lambda = 1.1, 1.2$, and 1.3 and loss factor (bottom) for different storage protocols under circumferential (left) and longitudinal (right) stretching. Raw measurements (dots) and mixed-effects model-based estimated marginal means (EMM) with 95% confidence intervals (CI) are shown side by side. Statistical significance relative to the *Control* group is indicated by * ($p < 0.05$) at each stretch level. The horizontal line and shaded band represent the control group EMM and its 95% CI, respectively.

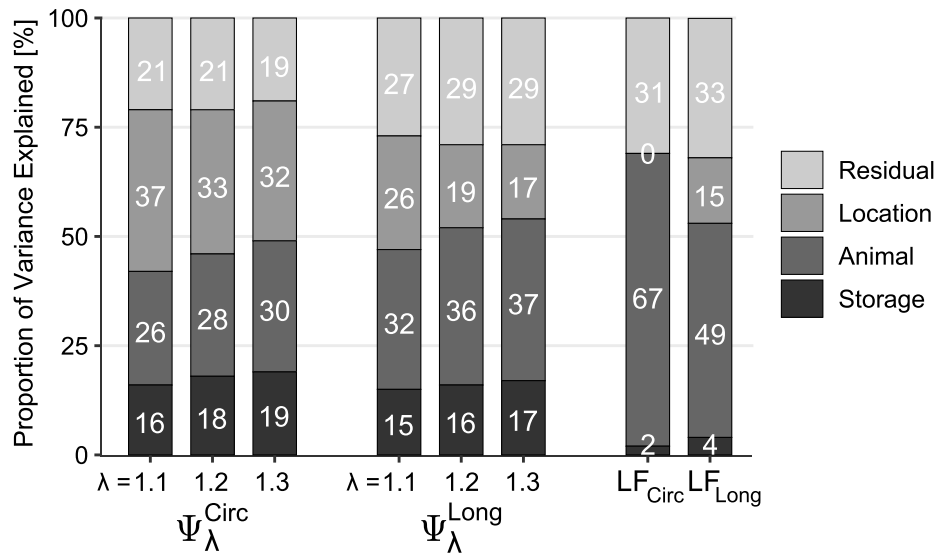


Fig. 4. Proportion of the total variance in the measured mechanical parameters explained by different factors, as determined by a linear mixed-effects model. The mechanical parameters include the strain energy density Ψ_{λ}^i and the loss factor LF_i , measured under loading protocol i (where $i = Circ$ for circumferential stretching, and $i = Long$ for longitudinal stretching) at stretch levels of $\lambda = 1.1, 1.2$ and 1.3 . Storage represents the different storage protocols. Animal accounts for inter-animal variability, while location refers to the excision site within the aorta, capturing intra-animal variability. The residual variance represents unexplained variability not accounted for by the model.

the smaller changes observed in the longitudinal direction reflect genuinely smaller effect sizes rather than increased variability, suggesting that storage-induced changes in this loading direction are comparatively modest. The observed difference between circumferential and longitudinal responses likely reflects the distinct roles of structural constituents, as circumferential behavior is primarily governed by collagen fibers aligned predominantly along this direction (Zeinali-Davarani et al., 2015; Schriebl et al., 2012), whereas longitudinal behavior is more influenced by elastin. Our findings may therefore suggest that the collagen and elastin components of the arterial wall extracellular matrix exhibit different sensitivities to cold storage. Consistent with this, mechanical changes due to storage were previously shown to associate more strongly with alterations in collagen compared to elastin architecture (Xing et al., 2015; Chow and Zhang, 2011).

4.1. Influence of storage temperature on elastic behavior

Refrigerated specimens exhibited significantly reduced strain energy density, regardless of the stretching direction, indicating tissue softening due to refrigeration. This change in elastic behavior can be attributed to the continued enzymatic and cellular degradation in the excised tissue during cold storage (Chow and Zhang, 2011; Hemmasizadeh et al., 2012; Wille et al., 2008), which was also linked previously to reduced tissue stiffness, stress, and strength (Chow and Zhang, 2011; Stemper et al., 2007; Hemmasizadeh et al., 2012; Adham et al., 1996).

Similar to refrigeration, freezing can impact tissue behavior due to the changes in both the cells and the extracellular matrix (ECM). At the cellular level, freezing has been shown to reduce cell viability and smooth muscle cell (SMC) contractility (Müller-Schweinitzer, 2009; Venkatasubramanian et al., 2010; Kostelnik et al., 2024), which can affect the arterial wall's active behavior. SMCs, however, have been shown to have negligible contribution to passive behavior in the aorta (Zou and Zhang, 2012). Hence, freezing-induced changes in passive behavior are likely primarily due to the alterations in the ECM. In particular, extracellular ice crystal formation and dehydration can disrupt collagen fiber organization and alter collagen–water interactions (Karlsson and Toner, 1996; Hemmasizadeh et al., 2012; Venkatasubramanian et al., 2006, 2010; Giannetti et al., 2025). This can lead to increased collagen aggregation, reduced inter-fibrillar spacing, and changes in fibril diameter and alignment (Venkatasubramanian et al., 2010; Han et al., 2009; Ozcelikkale and Han, 2016; Ding et al., 2015). Such microstructural changes may promote earlier collagen recruitment during loading by reducing the initial fiber crimp and increasing fiber engagement, thereby increasing apparent stiffness. Consequently, the increased strain energy density observed in most frozen groups is consistent with these structural alterations, although the magnitude of the effect depended on the stretch level, loading direction, and specific freezing protocol.

4.2. Influence of freezing protocol on elastic behavior

Freezing protocols have been associated with structural changes in the arterial wall, with slow and snap freezing triggering different mechanisms. Slow freezing promotes water migration and the formation of large extracellular ice crystals, which can disrupt both ECM and cells. In contrast, snap freezing rapidly solidifies water, producing smaller and more uniformly distributed ice crystals (Pegg et al., 1997; Karlsson and Toner, 1996; Mazur, 1996).

Snap freezing

Snap freezing is often assumed to better preserve tissue microstructure, hence also the tissue mechanical behavior. However, this assumption is primarily based on microstructural imaging studies with limited consideration of structure–mechanics relationships (Pegg et al., 1997; Karlsson and Toner, 1996; Mazur, 1996; Hoeier et al., 2025).

Despite this, evidence from cryobiology has shown that snap freezing can still induce microstructural damage, especially in thick or structurally heterogeneous tissues (Pegg et al., 1997; Karlsson and Toner, 1996). In the aorta, snap freezing was previously shown not to fully prevent structural damage. Delamination of the aortic wall has been observed in snap-frozen samples, where slow-frozen samples showed no such detectable damage (Kostelnik et al., 2024). In addition, elastin fragmentation has been reported (Rendal et al., 2004; Lőrinczy et al., 2023), which may influence passive mechanical behavior in snap-frozen samples.

Accordingly, studies investigating the mechanical consequences of snap freezing reported inconsistent findings for arterial wall. These discrepancies likely arise from methodological differences. Studies reporting no significant effects are typically characterized by small sample sizes and do not account for intra- and/or inter-subject variability (Bia et al., 2006; Rosset et al., 1996; Kostelnik et al., 2024; Langerak et al., 2001). In contrast, when the data was adjusted for these effects together with a larger sample size ($n = 20$), a significantly stiffer elastic behavior was detected following snap freezing (van der Laan et al., 2024).

In our present study, snap freezing groups (S_0 , S_{DMSO} , and S_{IP}) consistently demonstrated a significant increase in strain energy density, most prominently in the circumferential direction. These effects were observed irrespective of cryoprotective use or pre-cooling in isopentane. However, isopentane pre-cooling attenuated the effect, with increases in strain energy density reaching statistical significance only at higher stretch levels. This likely reflects improved thermal contact and more uniform cooling, which may reduce, but not eliminate, freezing-induced alterations in microstructure (Pegg et al., 1997; Venkatasubramanian et al., 2010; McKenzie et al., 2024).

Slow freezing

Microstructural alterations such as randomly dispersed circular defects and micro-fractures have been reported (Kropatsch et al., 2024; Novotny et al., 2017) due to slow freezing to -80°C , although these changes have not been directly linked to changes in elastic behavior. While some of the studies examining the effects of slow freezing on tissue elastic properties found no significant changes (Pukacki et al., 2000; Kostelnik et al., 2024; Hemmasizadeh et al., 2012; O'Leary et al., 2014; Stemper et al., 2007; Virues Delgadillo et al., 2010), others reported stiffer material response after slow freezing (Blondel et al., 2000; Venkatasubramanian et al., 2006; Chow and Zhang, 2011; Thakrar et al., 2006), especially if a cryoprotective agent was used (Blondel et al., 2000; Venkatasubramanian et al., 2006; Thakrar et al., 2006). Consistent with the latter group of studies, our results show that slow freezing without cryoprotectant (F_{PBS}) does not substantially alter elastic properties, whereas inclusion of a cryoprotective agent (F_{DMSO}) produces measurable changes, highlighting its influence on tissue mechanics.

4.3. Influence of cryoprotectant use on elastic behavior

Previous studies reported contradictory effects of cryoprotective agents on arterial mechanics. For snap freezing, the few available studies consistently reported no significant effects of cryoprotectant use (Kostelnik et al., 2024; Langerak et al., 2001). For slow freezing, some studies also reported no significant changes (Pukacki et al., 2000; Kostelnik et al., 2024; Kovács et al., 2025; van Doormaal et al., 2014; Virues Delgadillo et al., 2010; Adham et al., 1996; Bia et al., 2006). Notably, studies reporting no significant differences rarely employed paired analyses (Kovács et al., 2025; Langerak et al., 2001), potentially limiting sensitivity to detect small-magnitude but meaningful differences. In contrast, some other studies demonstrated increased stiffness after slow freezing with a cryoprotectant (Rosset et al., 1996; Venkatasubramanian et al., 2006; Blondel et al., 2000). Additionally, although not specifically on elastic behavior but on a mechanical tissue feature, fracture-focused studies more consistently demonstrated altered failure

behavior following cryoprotectant use (Dahl et al., 2006; Elder et al., 2005; Thakrar et al., 2006).

In our results, protocols with a cryoprotective agent (F_{DMSO} and S_{DMSO}) resulted in significantly increased strain energy density compared to fresh controls. While cryoprotective agents mitigate intracellular ice formation during freezing and may limit ice-induced microstructural changes (Tas et al., 2021; Venkatasubramanian et al., 2006), they can also affect collagen–water interactions. By penetrating hydration shells and disrupting water-mediated intermolecular bonding (Lee and Baiz, 2022; Cheng et al., 2015), cryoprotectants may reduce fibril mobility and constrain collagen fibers into a stiffer configuration, thereby increasing tissue stiffness.

When a cryoprotective agent was used, we observed no significant differences between slow and snap freezing (F_{DMSO} vs. S_{DMSO}). This may indicate that the presence of a cryoprotective agent is a more dominant factor affecting the mechanical response than the freezing rate. It is also noteworthy that the snap-frozen samples without a cryoprotectant agent (S_0) in our study showed strain energy density comparable to the samples stored with a cryoprotective agent (S_{DMSO}). Snap-frozen samples without a cryoprotectant agent are more susceptible to cryoinjury, which may also increase stiffness through microstructural disruption and explain the comparable elastic behavior change observed.

Overall, these effects are likely influenced by incomplete removal of the cryoprotective agent after thawing. Although washing protocols of approximately 20–30 min are commonly used (Gatto et al., 2013; Blondel et al., 2000; Pukacki et al., 2000; Adham et al., 1996), complete removal of cryoprotectants from arterial tissues remains difficult (Díaz Rodríguez et al., 2017; Hu and Wolfenbarger, 1994; Gatto et al., 2013). In addition, DMSO is known to be cytotoxic at room temperature (Fahy, 1986) and requires handling in a fume hood, which imposes practical constraints on its use. To limit cytotoxic exposure, samples in the present study were rinsed in PBS for approximately 10 min. Given the limited rinsing time and the known difficulty of fully removing DMSO from arterial tissue, it is likely that residual cryoprotectant remained within the samples and contributed to the observed mechanical response. The mechanical impact of residual cryoprotectant, together with the handling requirements of DMSO, supports simpler storage approaches as a more practical and reliable option for standard biomechanics laboratories without cryopreservation expertise.

4.4. Impact on viscous properties

Previously, a small but significant decrease in energy loss after 48 h of refrigeration was reported (Hemmasizadeh et al., 2012), suggesting that short-term storage can influence aortic viscous properties. This is broadly consistent with our findings, in which energy loss was slightly lower in refrigerated samples compared to fresh tissue, although the change did not reach statistical significance. Given that energy dissipation in elastic arteries such as the aorta is inherently low, subtle reductions may be challenging to resolve within experimental variability. Nevertheless, given the relatively large sample size per group ($n = 27$), our results indicate that short-term storage at $+4^\circ\text{C}$ has a limited effect on aortic viscous behavior.

Similarly, for frozen samples, we observed no significant differences in viscous properties compared to fresh samples, as also reported before by others for elastic arteries (Hemmasizadeh et al., 2012; Bia et al., 2006; Pessana et al., 2004; Kostelnik et al., 2024). However, this finding may not apply to the arteries of more muscular type as shown previously (Thakrar et al., 2006), highlighting that freezing-induced changes in viscous behavior are tissue-specific (Venkatasubramanian et al., 2006; Liu et al., 2020; McManamon et al., 2020).

Moreover, we did not find a significant effect of cryoprotectant use on viscous properties with respect to fresh tissue, which is in agreement with previous studies reporting preserved energy dissipation following

cryoprotectant-based storage (Shahmansouri et al., 2015; Venkatasubramanian et al., 2006; Virues Delgadillo et al., 2010; Kostelnik et al., 2024).

Overall, viscous dissipation was largely preserved across storage groups, with no statistically significant differences relative to the control condition. Although the $RFG-F_{PBS}$ contrast reached significance, the corresponding mean LF values (approximately 3.2–3.9%) remained close to the control group mean (approximately 3.5%), indicating no meaningful deviation in viscous behavior. The other frozen and snap-frozen groups showed LF values comparable to fresh tissue, and cryoprotectant use did not produce detectable changes in viscous behavior. Together with the variance decomposition, which indicated that storage effects contributed minimally to overall LF variability, these findings suggest that viscous properties of the aortic wall are only modestly influenced by the storage protocols examined.

4.5. Variability among animals and anatomical locations

The use of mixed-effects models in the statistical analysis allowed us not only to account for but also assess the impact of variability on arterial wall viscoelastic behavior. While some residual variance remains (possibly due to measurement noise, protocol deviations, and remaining unmodeled effects Famaey et al., 2026), our analysis indicated that inter-animal and anatomical location variability are major contributors to the observed mechanical response, even exceeding the influence of storage protocol.

Consistent with the variance partitioning results (Fig. 4), storage-induced changes per storage protocol were generally smaller than the baseline biological variability associated with animal identity and anatomical location. The notable exception was refrigeration (RFG), for which the storage-induced changes in mechanical parameters approached the magnitude of the biological standard deviation (approximately $0.8\text{--}1.0 \times \sigma_{bio}$). This may explain why refrigeration-related effects have been reported relatively consistently in the literature, even in studies that do not explicitly account for intra- and inter-animal variability. In contrast, the effects of the other storage protocols were substantially smaller relative to the underlying biological heterogeneity and may therefore be more difficult to detect without study designs that explicitly account for this variability.

These findings underscore the dominant role of biological heterogeneity in viscoelastic tissue behavior. As a result, studies that do not explicitly account for this dependency may either overlook meaningful changes or attribute variability in results to storage that is in fact biological in origin. This highlights the need for hierarchical statistical approaches, such as mixed-effects models or paired analyses, to disentangle experimental effects from intra- and inter-subject variability.

4.6. Practical implications

From a practical perspective, the present results suggest that slow freezing in PBS to -80°C provides the most consistent preservation of fresh tissue behavior among the tested storage protocols. In contrast, other storage methods were associated with measurable alterations in elastic properties, particularly in the circumferential direction, which should be considered when interpreting mechanical outcomes.

In particular, although they may be acceptable in some contexts, alternative storage approaches were consistently associated with changes in elastic behavior, which may also indirectly influence other mechanical outcomes as these are often not fully independent of the underlying elastic response. Notably, slow freezing in PBS is a relatively simple storage protocol compared to protocols involving liquid nitrogen or cryoprotectants such as DMSO, making it readily implementable and potentially contributing to improved consistency across laboratories.

4.7. Study design considerations

First, only porcine descending aorta was investigated; while structurally similar to human aorta, caution is required when interpreting our findings for human arteries or the ones of other species. Secondly, the descending aorta is an elastic artery; the obtained results may not generalize to muscular arteries and other vascular beds. Third, all specimens were obtained from healthy animals, whereas pathological tissue may respond differently to storage protocols.

Although group sample sizes were relatively large, the study may still be underpowered to detect very small effects, particularly for viscous responses, which are inherently low in elastic arteries. This may also apply to elastic behavior in the longitudinal direction, where effect sizes were smaller than in the circumferential direction despite comparable precision. Any undetected longitudinal effects are therefore likely to be small and of limited practical relevance.

Next, storage duration was not systematically evaluated. While frozen storage beyond the initial freezing event appears to have minimal additional impact (O'Leary et al., 2014; Kovács et al., 2025; Lőrinczy and Fazekas, 2022; Lőrinczy et al., 2023), refrigeration effects are more time-dependent. In the present study, samples were refrigerated for one week, which may represent a relatively prolonged duration. Earlier work has reported measurable mechanical alterations within 48 h of refrigeration, suggesting that this approach is only appropriate for short-term storage (Stemper et al., 2007; Ghosn et al., 2011; Adham et al., 1996; Chow and Zhang, 2011).

No microstructural or histological analyses were performed, preventing direct confirmation of mechanisms such as ice-crystal formation, dehydration, or collagen aggregation. Similarly, tissue water content was not measured, limiting assessment of osmotic effects. Although no clear thickness changes were observed in frozen samples, swelling of approximately 10% occurred in refrigerated specimens. Use of osmotically balanced preservation solutions (e.g., Euro-Collins, UW solution) may mitigate such effects (Ghosn et al., 2011).

The constitutive analysis assumed incompressibility, as is standard in arterial biomechanics. Potential storage-induced changes in tissue's water content could therefore influence estimated parameters through deviations from this assumption. Direct mass measurements would be required to separate intrinsic material changes from hydration-related effects.

Protocols involving DMSO introduce additional uncertainty. Complete removal of DMSO before testing cannot be guaranteed, and residual amounts may contribute to stiffening. Longer rinsing times were limited by concerns about cytotoxicity and the lack of temperature-controlled rinsing equipment, highlighting practical challenges in standardizing DMSO-based protocols.

Finally, although a consistent thawing protocol was applied to all samples (thawing at +4 °C followed by immersion in PBS at room temperature), the influence of thawing conditions on mechanical behavior was not examined. This is an important consideration, as prior studies indicate that thawing methodology can significantly affect tissue mechanics (Pegg et al., 1997; Müller-Schweinitzer et al., 2007). Therefore, while the protocol used here aligns with commonly recommended practices favoring slow thawing over rapid methods such as immersion in a hot water bath (Rendal et al., 2004; Novotny et al., 2017), no conclusions can be drawn regarding its optimality in maintaining the passive mechanical properties of arterial wall.

5. Conclusion

This study systematically evaluated the impact of six commonly used storage protocols on the viscoelastic behavior of the arterial wall by testing porcine descending aorta specimens under multi-directional loading.

We demonstrated that, while viscous properties remain relatively insensitive to storage conditions, cold storage alters the arterial wall's

elastic properties, particularly in the circumferential direction. Snap freezing and cryoprotectant use introduced protocol-dependent increases in tissue stiffness, reflected by increased stored strain energy density. Slow freezing in PBS to −80 °C and storage at this temperature best preserved tissue behavior, yielding mechanical responses closely comparable to fresh arterial wall. Taken together, these findings suggest that slow freezing in PBS provides the most consistent approximation of fresh tissue behavior among the tested methods. Furthermore, we showed that biological variability arising from differences in animal identity and anatomical locations within the aorta also significantly influences the arterial wall's mechanical behavior.

Overall, our study provides a practical guide for selecting an appropriate storage strategy in arterial wall mechanical research and highlights the critical role of biological variability in arterial wall passive behavior.

CRediT authorship contribution statement

Silke Dreesen: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kristyna Holko:** Writing – review & editing, Visualization, Methodology, Data curation, Conceptualization. **Maifarah Anthonijsz:** Writing – review & editing, Methodology, Conceptualization. **Ali C. Akyildiz:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at <http://dx.doi.org/10.1016/j.jmbbm.2026.107541>.

Data availability

Data will be made available on request.

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