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# Effect of Freezing and Thawing on the Biomechanical Characteristics of Porcine Ocular Tissues

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*All authors were fully involved in the study and preparation of manuscript and the material within has not been and will not be submitted for publication elsewhere.*

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**Abstract**

**Purpose:** To evaluate the effect of freezing and thawing on the biomechanical properties of ex-vivo porcine ocular tissue.

**Methods:** Thirty-six porcine eyes (18 pairs) were obtained fresh from a local abattoir and split into two groups of nine pairs to study the effect of storage at -20°C and -80°C. A randomly-selected eye from each pair (Control Group, CG) was tested fresh while the fellow eyes were frozen for 14 days, either at -20°C and -80°C (Frozen Group, FG) before thawing and testing. Seventy-two strips were extracted from the corneas and scleras of eye globes and subjected to uniaxial tension tests under loads up to 1.0 N. Following five preconditioning cycles, the load and elongation data obtained experimentally were analysed to derive the tissue's stress-strain and tangent modulus-strain behaviour.

**Results:** Corneal tissue subjected to freezing at -20°C exhibited significant increases in tangent modulus (mechanical stiffness) by 13±17% ($p= 0.003$) at 1% strain and 14±12% ($p< 0.001$) at 2% strain. In contrast, the increases in corneal stiffness at -80°C were insignificant (6±14%, $p= 0.099$ at 1% strain, 6±15%, $p= 0.091$ at 2% strain). The corresponding increases in tangent modulus in the sclera were all insignificant (for -20°C: 4±14%, $p= 0.265$ at 1% strain, 3±9%, $p= 0.186$ at 2% strain; for -80°C: 3±18%, $p= 0.537$ at 1% strain and 3±18%, $p= 0.491$ at 2% strain).

**Conclusions:** The study provided evidence that freezing and thawing led to insignificant changes in ocular tissue stiffness except in corneal tissue that was frozen at -20°C.

## Introduction

The ocular tunic, that includes the cornea and sclera, is a pressurised collagenous vessel of varying thickness and curvature. The soft and transparent structure of the cornea allows it to deform and act as a buffer to fluctuations in intraocular pressure (IOP) while being responsible for more than two thirds of the eye's refractive power (Dubbelman et al., 2006). On the other hand, the sclera's higher stiffness is necessary for maintaining the eye shape and the relative location of the main ocular components (Jesus et al., 2017) (Oyster and Haver, 1999). The stiffness of both components has been shown to increase with age due to a process known as non-enzymatic crosslinking (Geraghty et al., 2015) and to change with conditions including diabetes, keratoconus and myopia (Vinciguerra et al., 2016). The importance of tissue behaviour in maintaining clear vision as well as the implications resulting from age- and disease-related changes has led to increasing interest in this field.

Storage of soft tissues in freezing conditions before use in laboratory biomechanical testing has been a common practice since the 1950s (Coriell et al., 1964). The preservation process starts soon after cessation of circulation to avoid morphological distortions and damage due to drying, autolysis and putrefaction. Unlike cryotherapy or microscopic based studies where cell damage needs to be avoided (Valencia and Malacara, 2013, Fullwood and Meek, 1994, Chi and Kelman, 1966, Lai, 2015), the main focus in preserving tissue for mechanical testing is to keep the mechanical characteristics unchanged. While several studies have assessed the effect of freezing on the biomechanical properties of the brain (Metz et al., 1970), tendons (Chi and Kelman, 1966, Giannini et al., 2008), ligaments (Jackson et al., 1991, Moon et al., 2006, Woo et al., 1986), meniscus (Lewis et al., 2008) and arterial tissues (Burton et al., 2017, Delgadillo et al., 2010), the effect of this preservation method on the biomechanics of ocular tissues has received little interest. In recognition of the need to characterise the effects of freezing on ocular tissue, the present study considered two commonly used freezing

temperatures, namely -20°C and -80°C and investigated the effects on the tensile response of both corneal and scleral tissue. However, in light of the difficulties associated with obtaining human eyes for research, porcine ocular tissues were used for their similar tensile behaviour to human eyes (Zeng et al., 2001).

## Materials and Methods

### *Specimen preparation*

The study included 18 pairs of porcine eyes obtained fresh from a local abattoir. The eyes were separated into two groups of 9 pairs to study the effect of storage at -20°C and -80°C. From each pair, a randomly-selected eye was tested fresh less than 12 hours post-mortem while its fellow eye was stored in a freezer at the target temperature for 14 days before being thawed at room temperature and tested.

Two strips of tissue were extracted from each eye, one from the cornea and one from the sclera, using a custom-built, 4mm wide double-bladed cutting tool. Four cardinal points were marked around the limbus to help ensure strips were taken from the same locations for all eyes. Cornea strips were cut centrally in the nasal-temporal direction (the longest corneal diameter), while sclera strips were extracted from the superior quadrant of the anterior sclera approximately 1mm from the limbus, **Error! Reference source not found.**. While the majority of the sclera's collagen fibril orientation is reported to be random (Pijanka et al., 2013, Pijanka et al., 2014), a more circumferential organisation has been reported around the limbus. Therefore, the sclera strip extraction position has been selected close to the limbus in order to reduce uniaxial behaviour differences as much as possible due to variations in microstructure from one eye to another (Geraghty et al., 2012).

***Experimental setup***

Strip specimens were gripped using custom-built clamps and their initial length was accurately measured after clamping using a digital Vernier calliper (D00352, Duratool, Taiwan) accurate to ±10 μm. Cornea and sclera strips had an initial test length (distance between clamps) of 6 mm and 12 mm, respectively. Specimen width was measured at three equally-spaced locations along the test length, and averaged. The thickness was also measured using a Pachmate® DGH 55 ultrasonic pachymeter (DGH Technology, Exton, USA) with accuracy of ±5 μm at the same three locations and averaged. After gripping the specimens, the clamps were mounted on an Instron 3366, dual-column, table-top materials testing machine equipped with a calibrated 10 N load cell, Figure 2. Uniaxial tensile tests were carried out using a modular, Bluehill3 software package.

Specimens were subjected to five cycles of loading and unloading between 0.01 N and 1.0 N with a strain rate of 1.0 $mm.min^{-1}$ to precondition the tissue and stabilise its behaviour (Geraghty et al., 2012). A recovery period of 360 s was allowed between each two cycles to enable recovery of specimen's initial geometry and ensure the behaviour was not affected by the strain history of preconditioning cycles (Carew et al., 2000). Preconditioning was followed by a further application of load, up to 1.0 N, with the same strain rate, the results of which were considered representative of stable specimen behaviour. The required time to carry out a complete test varied between specimens but had a mean value of 40±5 minutes. Specimens were kept hydrated during the experiments using a phosphate-buffered saline (PBS) filled chamber which surrounded the clamps. Load-extension data was recorded by the Bluehill3 software, stored as comma-separated files (CSV) and processed by Microsoft Excel, **Error! Reference source not found.**3.

***Data processing***

Initial specimen slack was removed by applying a pre-load of 0.01 N. At this point 0.01 N was subtracted from all load values and the value of elongation at this load was also added to the initially measured length. The mechanical tensile stress $\sigma$ was calculated by dividing the applied load $F$ by the strip cross-sectional area $A$ (Davis, 2004) as

$$\sigma = \frac{F}{A} \qquad \textit{Equation 1}$$

The strain was calculated as the ratio of change in the strip extension $\Delta L$, which is the absolute difference between the initial strip length $L_0$ and its instantaneous length, $L$, at the time of calculating the strain, over the initial length.

$$\varepsilon = \frac{\Delta L}{L_0} \qquad \textit{Equation 2}$$

Since hyperplastic materials have a nonlinear stress-strain relationship, their stiffness should be expressed by the tangent modulus. To obtain the tangent modulus accurately, the stress-strain curve needs to be smooth and noise in data must be removed. Hence, the stress-strain data was fitted to an exponential equation ($\sigma = A(e^{B\varepsilon} - 1)$) similar to the approach adopted in earlier studies (Elsheikh et al., 2010). The tangent modulus $E_t$ was then calculated as the first derivative of the exponential stress-strain curve.

$$E_t = \frac{d\sigma}{d\varepsilon} = AB\, e^{B\varepsilon} \qquad \textit{Equation 3}$$

Following the same procedures for each fresh and frozen-thawed specimen, a tangent modulus ratio $R_t$ can be calculated at any stress or strain level as

$$R_t = \frac{E_{t\,(Frozen)}}{E_{t\,(Fresh)}} \qquad \textit{Equation 4}$$

### *Statistical analysis*

Matlab Statistics and Machine Learning Toolbox® (MathWorks, Natick, USA) was used to carry out the statistical analyses in this study. The paired two-sample T-test was used to compare two data sets to show whether they came from the same continuous distribution at 80% confidence level (statistical power β=0.2). This test was chosen as the data were normally distributed. The probability p, which is an element of the period [0,1], was determined where values of p> 0.05 indicate the validity of the null hypothesis, otherwise, it indicates the significance of the phenomenon (Everitt and Skrondal, 2010).

## Results

The average cornea strip dimensions for thickness, width and length were 1.024±0.071 mm, 4.597±1.081 mm and 6.655±0.514 mm, respectively. The corresponding values for sclera strips were 0.868±0.167 mm, 3.978±0.415 mm and 12.318±0.334 mm, respectively. Visual inspection of specimens during testing showed that the effects of the end clamps (in preventing reductions in tissue width) became evident only near the end of the loading range but were not notable at the low stress levels (about 0.02 MPa stress) at which the tangent modulus was calculated.

All cornea and sclera specimens exhibited nonlinear, hyper-elastic behaviour with an initial low stiffness (tangent modulus, Et), which increased gradually with increasing stress, Figure 4. The pattern of hyperelastic behaviour exhibited by both fresh and frozen specimens was similar to that reported for soft biological tissue in earlier studies and appeared not to have been affected by freezing (Elsheikh et al., 2007, Anderson et al., 2004, Geraghty et al., 2012, Zeng et al., 2001, Elsheikh et al., 2008, Fung, 1993). The results further show gradually

diminishing changes in behaviour from one cycle to another, justifying the use of the last cycle as representative of specimen behaviour. The exponential form of the stress-strain form further meant that the $E_t$-σ relationship was linear (as $E_t = B(\sigma + A)$ while $E_t$-ε remained exponential ($E_t = ABe^{B\varepsilon}$).

The ratio of tangent modulus of frozen and fresh tissues ($R_t$) at specific strain and stress levels was used as a quantitative measure of the changes in tissue stiffness as a result of freezing and thawing. Due to physiological and mechanical variations among eyes taken from different animals, the comparisons in this study were limited to same animal fresh and frozen eyes. The results shown in Table 1 show that significant increases in tissue stiffness were only found in cornea specimens stored at -20°C (p= 0.003 and <0.001 at 1% and 2% strain, respectively). With the higher freezing temperature of -80°C, there were no significant changes in corneal Et at both strain levels (p> 0.05). On the other hand, scleral tissue did not show any significant changes in Et with freezing at both -20°C and -80°C (p> 0.05). In addition, significant differences were found in Et values between corneal specimens frozen to -20°C and -80°C (p=0.006), and in corresponding scleral specimens (p=0.015). However, these results were not based on the paired analysis conducted in this study, which was limited to comparing fresh specimens to frozen tissue with either -20°C or -80°C.

The Et ratios between fresh and frozen specimens including means, standard deviations and minimum and maximum values are presented in Figures 5 and 6 for cornea and sclera tissues, respectively. While mean Et ratios over the strain range of 0 - 0.3 in cornea specimens that were frozen to -20°C and -80°C were 1.22±0.19 (p= 0.000) and 1.07±0.14 (p= 0.021), respectively, the corresponding ratios in sclera specimens were 1.04±0.12 (p= 0.176) and 1.03±0.19 (p= 0.537). However, cornea specimens frozen at -20°C exhibited

different behaviour that was dependent on strain in an almost linear fashion (Et frozen/fresh ratio = $3.15\,\varepsilon + 1.08$). On the other hand, all other specimen groups showed Et ratios that were largely independent of strain.

## Discussion

This study aimed to provide a thorough analysis of the effect of freezing on the biomechanics of the cornea and sclera in porcine eyes. In the past, no changes were assumed in biomechanics and the material parameters of frozen tissues were considered to be equivalent to those of fresh specimens (Twa et al., 2014) (Schultz et al. (2008). This assumption, while being unproven in earlier studies, removed the need to test the tissue in a fresh condition and hence made the test protocol significantly easier. In the current study, it was found that the tangent modulus of corneal tissues undergoes significant increases (or around 13%) after freezing to -20°C. With freezing corneal tissue at -80°C or freezing scleral tissue at either -20°C or -80°C, the increases in tangent modulus were not significant. In addition to these results, significant differences were found in both corneal and scleral specimens between freezing to -20°C and -80°C.

The strip testing adopted in this study is a simple technique with few steps to prepare the specimens and analyse the results. However, the technique has a number of limitations caused by the initially curved form of the specimen and the termination of fibrils along the specimen sides (Elsheikh and Anderson, 2005, Hoeltzel et al., 1992). The straightening of the specimens from their curved form results in initial strains that affect the behaviour under subsequent loading. The relatively large specimen thickness poses another difficulty with the potential of unequal clamping of external and internal tissue layers. The uniaxial loading adopted in strip testing is also different from the biaxial loading expected in intact eye globes,

leading possibly to changes in obtained behaviour. Although these limitations affect the technique's suitability to determine the global properties of ocular tissue, the technique remains viable for comparative studies, such as the present research where the focus is on the variation in tissue behaviour caused by freezing and thawing. Several studies have in the past successfully used the strip testing technique to address similar challenges and answer specific questions on the effect of certain parameters on tissue behaviour (Seiler et al., 2018, Xue et al., 2018, Zhang et al., 2018, Richoz et al., 2014, Kling et al., 2012).

The use of animal eyes as approximate models for human tissue in mechanical property characterisation studies such as the current research had been necessary because of the difficulties in obtaining human donor eyes in sufficient numbers. Several earlier studies (Lombardo et al., 2006, Anderson et al., 2004, Voorhies, 2003, Kampmeier, 2000, W.Nyquist, 1968) used porcine eyes and relied on earlier comparative analysis of human and porcine eyes in which porcine specimens were found to have similar stress-strain behaviour to human specimens (Zeng et al., 2001, Elsheikh et al., 2008).

The present study on the effect of freezing on ocular tissue relied on behaviour comparisons between pairs of eyes obtained from the same animals, with each pair including an eye that was tested fresh and another tested after freezing and thawing. This methodology relied on earlier evidence that eyes of the same animal exhibited similar behaviour while eyes of different animals showed a wide range of behaviour patterns and stiffness values (Elsheikh and Alhasso, 2009).

The mean ratio of tangent modulus ($E_t$) between -20°C frozen and fresh corneal tissue was dependent on strain in an almost linear form while other specimen groups did not show any significant dependence on strain. However, these observations are only valid for the locations and orientations of the specimens tested in this study, and different behaviour may be

obtained if these were to change. Nevertheless, the study presented clear evidence that corneal tissue frozen at -20°C has behaved differently from corneal tissue frozen at -80°C and scleral tissue frozen at -20°C or -80°C. This difference was evident in larger $E_t$ increases, that were statistically significant, and $E_t$ ratios that were dependent on strain.

The study involved a number of limitations, some of which have been discussed above. Further, the study considered particular specimen locations and orientations, a particular freezing period (14 days) and a particular strain rate in testing (1.0 $mm.min^{-1}$). Nevertheless, while it is acknowledged that changes in these parameters may lead to different outcomes, it is not expected that the differences will be significant as the characteristics of collagen fibrils (the main load carrying components of corneal and scleral tissue) do not show large regional variations (Meek, 2008, Boote et al., 2006). Moreover, while this study demonstrated that the corneal tissue stiffness has increased as a result of freezing at -20°C, it did not demonstrate the underlying causes, which are most likely related to changes in tissue microstructure.

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**Declaration of interest:**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**Contributions:**

AA drafted manuscript and interpreted results, A. Eliasy supervised experiments, collected results and helped with drafting the manuscript, BG provided training for lab experiments and reviewed the manuscript, ME and AH conducted experiments, A. Elsheikh designed and

supervised the entire project, all authors have reviewed the manuscript, approved the final draft and had significant contribution to the study.

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## Figure captions

Figure 1 Right eye schematic showing cornea and sclera specimen locations. N = nasal, T = temporal, S = superior, I = inferior, A = apex, PP = posterior pole

Figure 2 (a) Custom-made clamps for uniaxial experiments on cornea or sclera strips, (b) A specimen being clamped and hydrated prior to the experiment

Figure 3 Variation of load over time in a test involving a typical cornea specimen under cycles of loading and unloading

Figure 4 (a) Load-extension behaviour of a typical cornea specimen under loading cycles, (b) stress-strain behaviour based on the load-extension behaviour of the last loading cycle, (c, d) variation of tangent modulus with strain and stress during the last loading cycle

Figure 5 Ratio of tangent modulus in (a) -20°C and (b) -80°C frozen corneas relative to fresh cornea specimens

Figure 6 Ratio of tangent modulus in (a) -20°C and (b) -80°C frozen scleras relative to fresh sclera specimens

Table 1 Mean and standard deviation of tangent modulus ratios between frozen and fresh tissue specimens

| | | 1% strain | 2% strain |
|---|---|---|---|
| Cornea | -20°C | 1.130 ± 0.17 (p= 0.003) | 1.144 ± 0.12 (p< 0.001) |
| | -80°C | 1.061 ± 0.14 (p= 0.099) | 1.064 ± 0.15 (p= 0.091) |
| Sclera | -20°C | 1.037 ± 0.14 (p= 0.265) | 1.029 ± 0.09 (p= 0.186) |
| | -80°C | 1.027 ± 0.18 (p= 0.537) | 1.030 ± 0.18 (p= 0.491) |

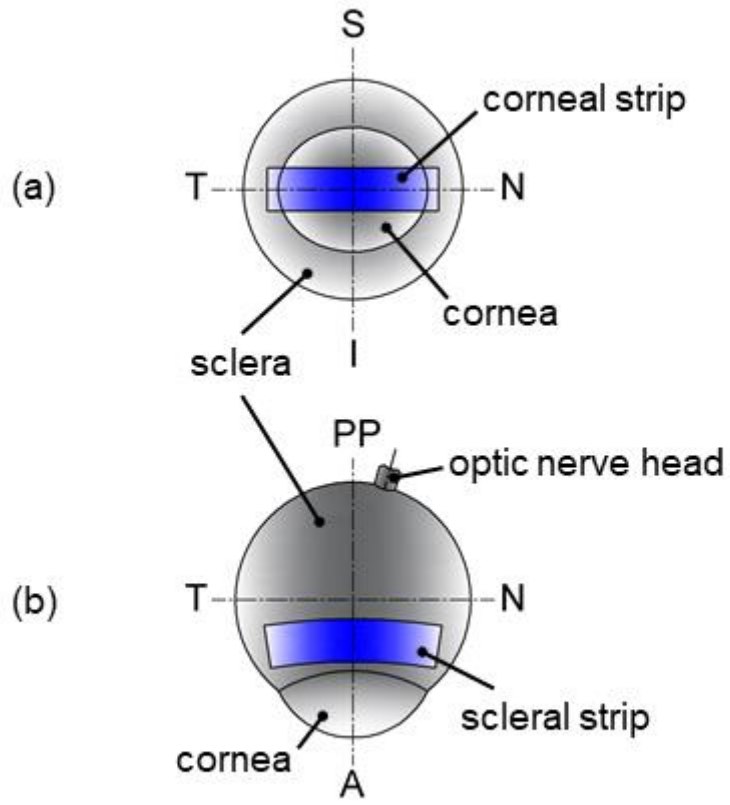


Figure 1: Right eye schematic showing cornea and sclera specimen locations. N = nasal, T = temporal, S = superior, I = inferior, A = apex, PP = posterior pole

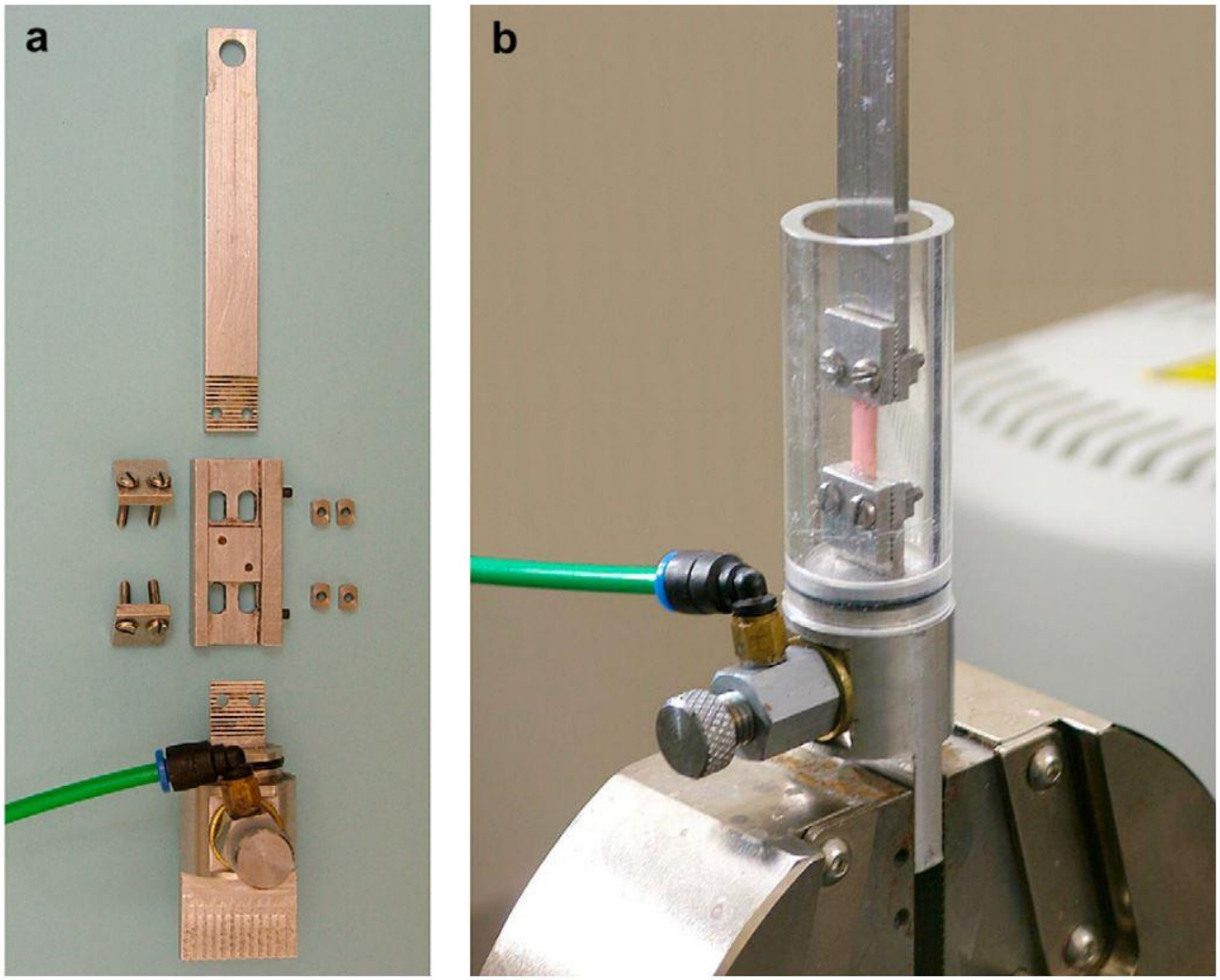


Figure 2: (a) Custom-made clamps for uniaxial experiments on cornea or sclera strips, (b) A specimen being clamped and hydrated prior to the experiment

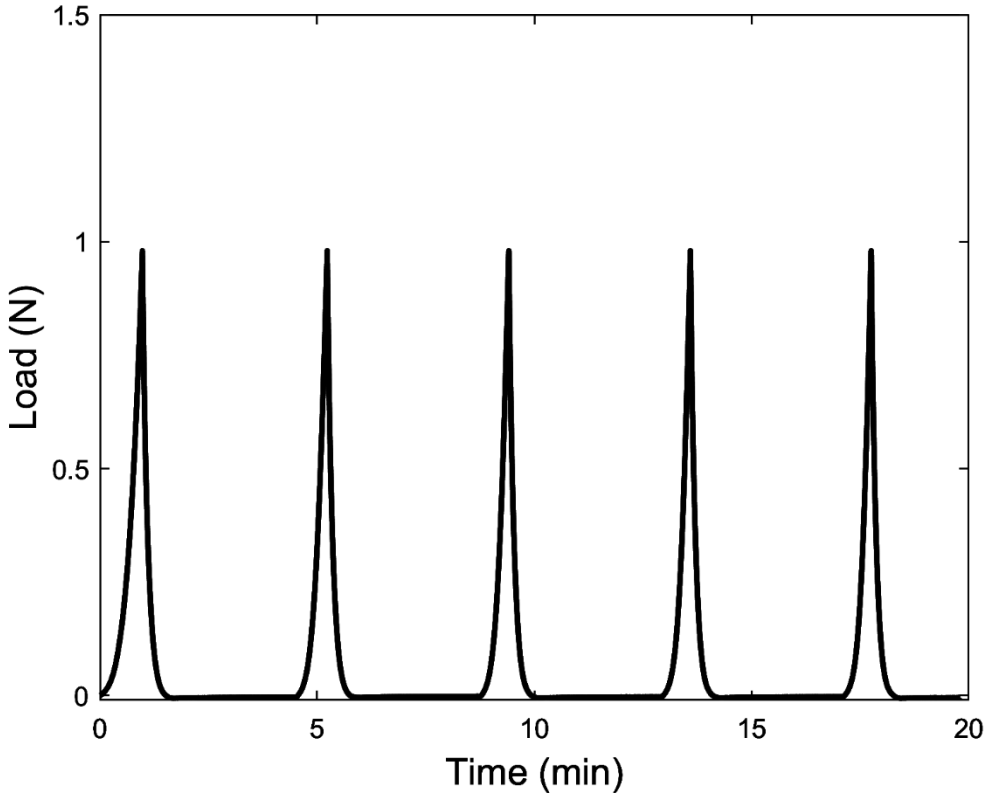


Figure 3: Variation of load over time in a test involving a typical cornea specimen under cycles of loading and unloading

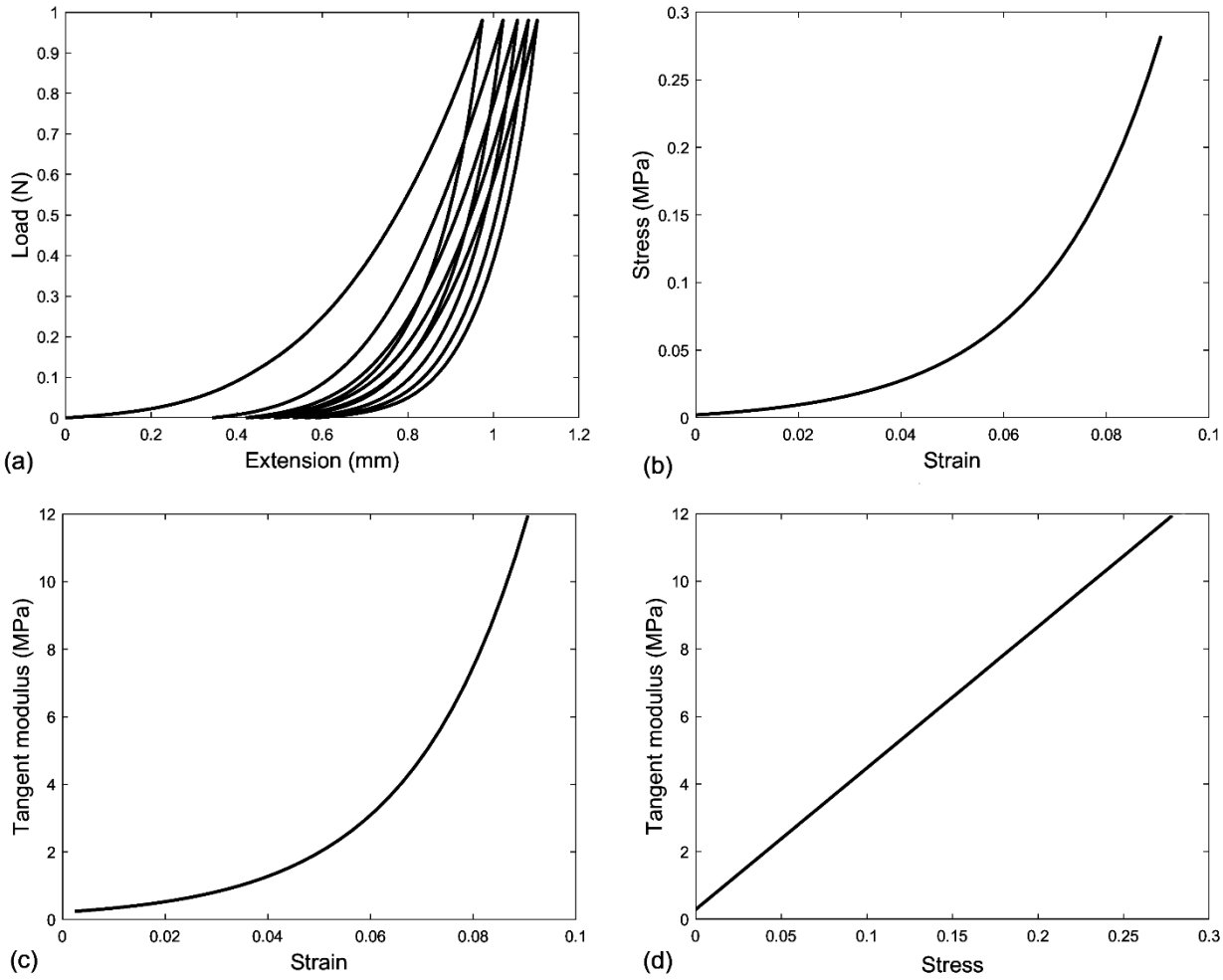


Figure: (a) Load-extension behaviour of a typical cornea specimen under loading cycles, (b) stress-strain behaviour based on the load-extension behaviour of the last loading cycle, (c, d) variation of tangent modulus with strain and stress during the last loading cycle

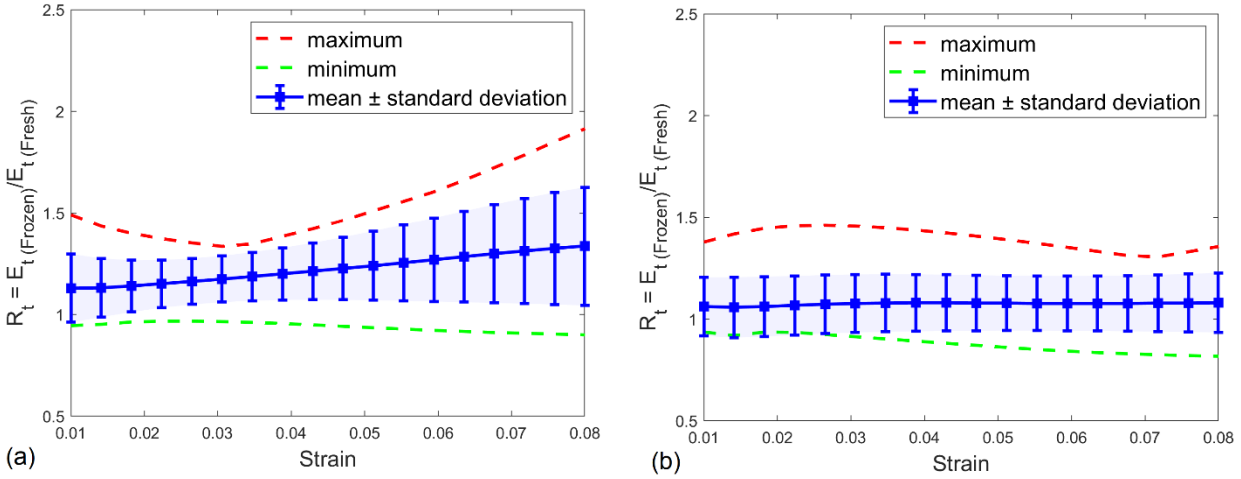


Figure 5: Ratio of tangent modulus in (a) -20°C and (b) -80°C frozen corneas relative to fresh cornea specimens

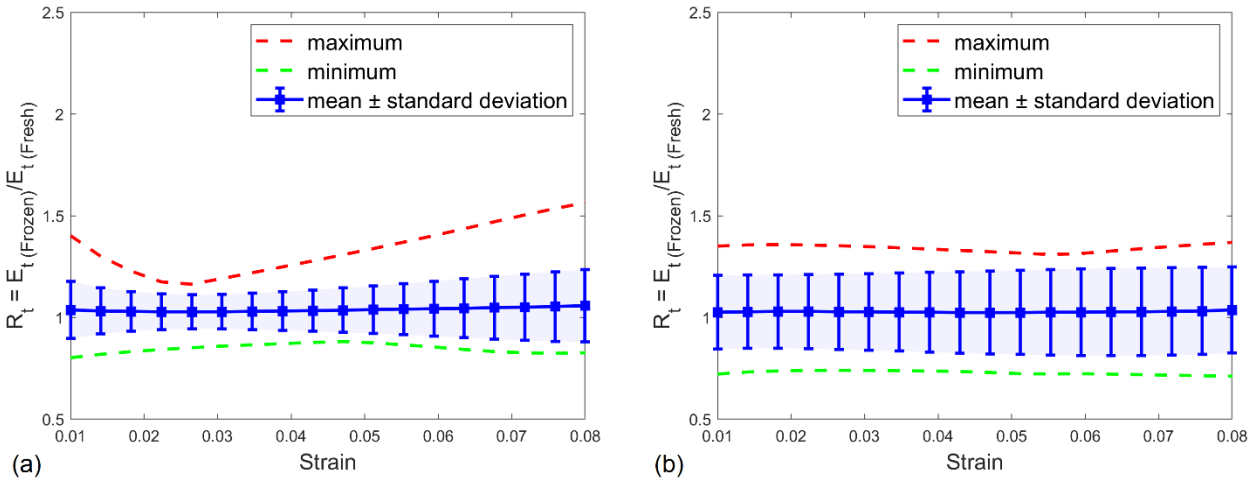


Figure 6: Ratio of tangent modulus in (a) -20°C and (b) -80°C frozen scleras relative to fresh sclera specimens