**Microscale assessment of corneal viscoelastic properties under physiological pressures**

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The micromechanical behaviour of the cornea is important for understanding and modelling of many ocular disorders. Whereas inflation testing has been utilised to determine the bulk mechanical properties of the cornea under physiological pressures, micromechanical testing has been limited to unpressurised corneal samples. In this study the micromechanical properties of pressurised porcine corneas were determined using oscillatory nanoindentation coupled with a custom inflation method. Inflation was conducted in phosphate buffered saline (PBS) and tissue culture (TC) solutions. The shear storage modulus (G′) and shear loss modulus (G”) was determined for corneas inflated corneas with PBS and TC. Central corneal thickness (CCT) was monitored during the inflation (0-60 mmHg). Elastic modulus (E) was also calculated and quantitatively mapped for corneas. The results showed that G′ at 15 mmHg was 86.18±16 kPa and 88.86+13.54 kPa inflated by PBS and TC respectively. These values increased 3.2 times in an approximate linear relationship to 60 mmHg. G” at 15 mmHg was 12.5±2.5 kPa and 13.54 +1.9 kPa inflated by PBS and TC respectively. G” increased 1.9 times in an approximate linear relationship to 60 mmHg. No significant change was noticed in viscoelastic properties of corneas inflated by TC for 4 hours whereas 4 hours of hydration on PBS affected the mechanical properties. The central corneal region was found to be stiffer than in peripheral region. Mapping of elasticity revealed a symmetrical distribution of properties that varied with inflation. Our method has potential for measurement of viscoelastic properties of corneas in conditions where there have been localised changes in mechanical properties such as keratoconus.

Keywords: corneal viscoelasticity; corneal elastic mapping; corneal tissue culture; nanoindentation’ porcine corneas

# **Introduction**

The cornea is the principle refractive element of the eye which has a natural curvature and is transparent. These properties of the cornea arise from it being under intraocular pressure (IOP). The structural and optical properties of the cornea can largely be attributed to the stromal layer, which represents around 90 % of the corneal thickness. The stromal layer is composed of collagen fibres which provide the cornea with mechanical properties and are also responsible for corneal transparency. Conventionally, in vitro experimental testing has been conducted on isolated corneal strips with uniaxial testing, requiring preconditioning to align the collagen fibres. Additionally, in such a test the corneal tissue is not under physiological pressure. The importance of considering the microstructure of the cornea under physiological pressures and how that influences the mechanical behaviour of the tissue has been highlighted in a recent study (Benoit et al., 2016). In that study, polarization-resolved second harmonic generation microscopy (P-SHG) was combined with inflation testing as a novel approach to capture both surface strain and the evolution of collagen fibril re-organisation with increasing pressure in human corneas. The organization of collagen lamellae was recorded across pressures from 12 mmHg – 48 mmHg. Benoit et al. demonstrated that the stromal microstructure is modified with increased IOP. Interesting, they demonstrated that the preferential orientation of collagen lamellae differed across different regions of the cornea as the pressure increased.

Given this insight into how the stromal microstructure is altered across different regions as pressure increases, we hypothesised that a micromechanical approach such as nanoindentation could be used to capture regional variations in the viscoelastic behaviour of the cornea with increasing pressure. Nanoindentation is powerful and established technique for measuring small volumes of material. In recent years, approaches have been developed to utilise nanoindentation for hydrated biological tissues (Labate et al., 2015; Last et al., 2012, 2010, 2009; Lombardo et al., 2012; Oyen, 2015). Whilst there are a number of challenges that need to be overcome when utilising commercial instruments for testing of hydrated and compliant materials such as hydrogels and biological soft tissues, research to date has mostly focussed on analytical methods or technique development for maintaining hydration whilst testing. A major gap in biomechanical testing of soft tissues is the ability to conduct micro-scale testing under physiological pressure. This is important not only because the fibrous architecture is completely different to tissue in its relaxed (unpressurised) state (Walton et al., 2015), but, as already demonstrated for the cornea, the mechanical response and anisotropy of the cornea is significantly altered at high physiological pressures (Benoit et al., 2016; Singh et al., 2016).

In this study, we have utilised oscillatory nanoindentation coupled with inflation testing. The oscillatory nanoindentation method has previously been used for mechanical characterisation of hydrogels (Akhtar et al., 2018, 2016) canine articular cartilage (Peters et al., 2017) and porcine skin (Moronkeji et al., 2017). One of the main advantages of using this approach is the ability of probing regional locations of the cornea. Hence, when coupled with inflation testing this approach has significant advantages over macroscale inflation testing that is more widely used in the literature (Boyce et al., 2008; Elsheikh et al., 2008; Whitford et al., 2016).

Here, we focus on porcine corneas and demonstrate how the shear storage and loss modulus vary with increasing IOP (0 to 60 mmHg) across the central cornea. The inflation testing is conducted in phosphate buffered saline (PBS) and tissue culture medium (TC) to explore the role of different hydrating media. Corneal swelling and mechanical properties are also considered in terms of hydration time. Finally, the micromechanical properties are mapped across different regions of the cornea demonstrating the full potential of our technique.

# **Materials and Methods**

**2.1. Sample preparation**

Thirty four fresh porcine eyes were obtained from a local abattoir shortly after slaughter. The pigs were aged between 5 and 6 months. The epithelium layer was peeled off from the corneas using a cotton-tipped applicator and tweezers. The corneas, with a 2 mm scleral ring, were subsequently dissected and placed in a specially designed artificial anterior chamber. The corneas were split into two experimental setups as summarised in Table 1.

**Table 1** Summary of the experimental setup used within the study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Experiment 1 | | Experiment 2 | | |
| Incubation solution | TC | PBS | PBS | PBS | PBS |
| Number of corneas (n) | 8 | 8 | 6 | 6 | 6 |
| IOP (mmHg) | 0 – 60 | 0 – 60 | 0 | 15 | 60 |
| Indented region | Central | | Central to limbus | | |
| Incubation period (hours) | 0, 4 | | 0 | | |
| No. of indents per IOP | 3 | | 49 | | |
| Acquired data | G′, Gʹʹ, E, tan(δ), CCT | | E | | |

TC: tissue culture, PBS: phosphate buffered saline, G′: shear storage modulus, Gʹʹ: shear loss modulus, E: elastic modulus, tan(δ): loss factor, CCT: central corneal thickness. Experiment 1: Micromechanical characterisation under pressure, Experiment 2: Micromechanical mapping under pressure.

**2.2. Experiment 1: Micromechanical characterisation under pressure**

Sixteen corneas were divided into two groups categorised by the solution used to inflate the samples: Tissue culture solution (TC-0h group; n=8) or phosphate buffered saline (PBS-0h group; n=8). An elevated reservoir was filled with either TC solution (CARRY-C, ALCHIMIA, Italy) or PBS (Sigma-Aldrich, Dorset, UK). The solutions were used to apply hydraulic pressure to the posterior surface of the corneas, simulating intraocular pressures (IOP) in the range 0 to 60 mmHg. The pressure was controlled by the height of the reservoir and measured using a pressure sensor (ABP series, Honeywell, USA). The experimental setup is shown in Fig. 1.

The micromechanical testing was conducted with a Nanoindenter G200 system with a DCM-II head (KLA-Tencor, CA, USA). A cylindrical flat punch tip with a 100 μm diameter (Synton-MDP Ltd, Nidau, Switzerland) was utilised with the DCM-II indenter head. A custom, dome-shaped corneal holder was used to hold the corneas in place. In principle, this setup enabled the viscoelastic properties of the cornea to be measured at any point on the cornea with the spatial resolution limited by the indenter tip radius (100 μm).

Oscillatory nanoindentation was conducted to determine the shear storage (G′) and shear loss modulus (G″) using a method which has been described in detail elsewhere (Akhtar et al., 2018, 2016).

The apex of the inflated corneas was indented 3 times at each IOP level. Each indent was performed with a 700 µm distance between each indent, thus covering a total distance of 1700 µm in the peripapillary region of the cornea. A pre-compression of 5 μm was chosen that provided sufficient contact of the tip with the corneal apex surface, which was also used in a previous study on soft tissue (Akhtar et al., 2016). An oscillation amplitude of 500 nm and testing frequency of 110 Hz (the resonant frequency of the indenter head) was selected for all experiments. The indenter tip was cleaned by driving into double-sided Scotch Tape (3M) after each set of 3 indents. The testing and tip cleaning at each pressure step took approximately 12 mins.

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Fig. 1. Schematic diagram which shows a sketch of DCM-II head and the experimental setup that was used to vary the IOP during the nanoindentation process. The cornea was placed at the same level as the pressure sensor and zero pressure level. The DCM-II head consists of coil/magnet assembly, capacitance gauge, leaf spring and oscillation transducer (with permission form KLA Instruments Group). The coil/magnet assembly generates controlled forces by using an electromagnetic system. The capacitance gauge is used for sensing displacement. Leaf springs are used to secure the indentation column for stability and maximum lateral stiffness. All of the testing was conducted in a closed compartment to minimise any external mechanical vibrations.

The elastic modulus was calculated using Equation 1.

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| --- | --- | --- |
|  |  | (1) |

where *ν* represents the Poisson’s ratio of the cornea which was assumed to be 0.4 based on a previous study (Ford et al., 2011).

Central corneal thickness (CCT) was measured at 0 mmHg and then in 5 mmHg increments up to 60 mmHg by using a Pachymeter (DGH 55 Pachmate, DGH Technology Inc, USA). The accuracy of the pachymeter used was suitable because the manufacturer’s specifications state that its accuracy is 5 µm. Both the mechanical measurements and the corneal thickness measurements at each IOP level were repeated after 4 hours for both groups to detect any time-dependent changes. The 4 hours timeframe was between the testing at 0 mmHg of the first experiment and the testing at 0 mmHg of the second experiment. The corneas were kept in the holder in a relaxed state immersed in the same solution that was used for inflation. By keeping the corneas in the holder ensured that we were able to indent the same area in each experiment.

All of the experiments were carried out at room temperature (approximately 22oC) and 45±2.1% relative humidity, as determined with a humidity-temperature meter (OMEGA engineering, USA). One drop (40 µl) of PBS (Sigma-Aldrich, Dorset, UK) was applied on the external surface of the corneas at every pressure step to maintain the hydration of the tissue.

**2.3. Experiment 2: Micromechanical mapping under pressure**

Twelve corneas were used to map the elastic properties across the surface of the cornea under pressure; 15mmHg (n=6), and 60mmHg (n=6). A further 6 corneas were used under unpressurised condition (0 mmHg). The samples were prepared using the same method as described in 2.1. The orientation of corneas (nasal-temporal and superior-inferior) were marked when the samples were fixed on the dome-shaped sampled holder. The corneas were inflated with TC media and indented in an array covering 49 indents. The same experimental settings as described earlier were used for all of the nanoindentation tests except that the pre-compression value was increased to 7 µm. This higher value was required to make better contact with corneal surface at the peripheral region. The indent matrix covered a square region of 9 x 9 mm; the centre of the matrix was on the apex of the cornea. The corneal elastic modulus was calculated from the shear storage modulus as described earlier. A MATLAB code (Mathworks Company, Natick, Massachusetts, USA) was used to create a contour map showing the distribution of the elastic modulus under IOP across the surface of the cornea.

**2.4 Statistical analysis**

All statistical analysis was carried out using OriginPro 2016 version 9.3 (OriginLab, USA). All data are expressed as mean values and standard deviation (mean ± standard deviation). In order to determine statistical significance for the changes in shear storage and loss modulus and CCT at each IOP, paired sample t-tests were used. In order to determine statistical significance for changes in shear storage and loss modulus and CCT for the two incubation solution groups, unpaired two sample t-test was used. For nonparametric data (micromechanical mapping data), the Mann-Whitney test was used. The significance level (α) was set as 0.05 for all tests.

# **Results**

**3.1. Micromechanical properties under IOP**

The shear storage modulus (G′), shear loss modulus (G″), loss factor (tan(δ)) and the calculated elastic modulus of the inflated porcine corneas are shown in Fig. 2. An approximate linear relationship was observed between viscoelastic shear moduli and the applied internal pressure; as the internal pressure increases, the viscoelastic shear moduli also increases (R2 > 0.9). The loss factor (ratio of G″/ G′) was initially very high in the unpressurised cornea (>0.6) but reduced to around 0.1 as the IOP increased, Fig 2C. Hence, the viscous behaviour of the cornea was significantly diminished with increasing IOP. Overall, the trends in G′, G″ and tan(δ) were comparable across both time points and for both hydrating solutions. Gʹ following 4 hours of hydration in PBS deviated most from the other tests, as evident in Fig 2A.

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Fig. 2. Micromechanical properties of the cornea under IOP (A) shear storage modulus (G′), (B) shear loss modulus (G”), (C) loss factor (tan(δ)), and (D) elastic modulus (E). Error bars represent standard deviation. (n =8/group). PBS-4h refers to the same corneas that were initially inflated by PBS and then retested after 4 hours. TC-4h refers to the same corneas that were initially inflated by TC and then retested after 4 hours.

The elastic modulus of inflated porcine corneas is illustrated in Fig. 2D. At normal physiological pressure of porcine eyes inflated with PBS, G′ is 86.19±16.6 kPa and E is 241.3±46.5 kPa.

Statistical analysis shows that there is no significant increase in G′ between PBS-group and TC-group, (PBS-0h) and (TC-0h), when they were initially tested (p ≥ 0.201). There was no statistically significant increase in G′ of corneas that were initially inflated by TC and the same corneas when retested after 4 hours (p ≥ 0.073). In contrast, corneas inflated by PBS behaved differently; the shear storage modulus was significantly increased (33.7%) for corneas when retested after 4 hours (p ≤ 0.00013). A significant difference in G′ was seen (25%) between corneas in different groups when retested after 4 hours (p ≤ 0.000035).

When comparing the viscous component for the corneas inflated by the solutions, it was found that G” was approximately 6 % higher in TC-0h group as compared to PBS-0h group. However, this difference was not statistically significant (p=0.221). Interestingly, there was no significant increase in G” of corneas initially inflated by TC (TC-0h group) and the same corneas when retested after 4 hours (TC-4h group), (p≥0.409). Unlike the corneas inflated by TC, a significant increase in G” was observed (25%) between corneas initially inflated by PBS (PBS-0h group) and the same corneas when retested after 4 hours (PBS-4h), (p ≤ 0.029). Non-significant difference in G” was detected between corneas in different groups when retested after 4 hours (TC-4h and PBS-4h groups), (p≥0.324).

The loss factor was 5 % lower between corneas of PBS-0h group and the same corneas when retested after 4 hours (PBS-4h), (p ≤ 0.0408). On the other hand, there was no statistical difference in the loss factor between the two time points for corneas inflated by TC (p≥0.1802). The loss factor of corneas in PBS-0h group was 6 % lower than that of corneas in TC-0h group but not statistically significant (p≥0.183). This difference became statistically significant when the corneas of both groups (PBS-4h and TC-4h) were retested (p ≤ 0.0391).

**3.2. Central corneal thickness**

In all the groups, central corneal thickness (CCT) decreased as IOP increased, as shown in Fig. 3. The extent of this change differed in each of the groups. Initially, CCT of the TC-0h group was decreasing gradually for the first three IOP values in a similar manner to the PBS-0h group. Subsequently, the difference in CCT between PBS-0h and TC-0h groups became statistically significant after 15 mmHg (about 1.6 % reduction), (p < 0.036), which correlates to more than 37 min in the inflating solutions. The results of retesting the corneas after 4 hours showed that CCT of the PBS-4h group significantly increased (approximately 9.3 %), as compared to corneas in the PBS-0h group. In comparison, CCT of the TC-4h significantly decreased (approximately 11 %) than when the corneas were initially tested. The maximum difference in CCT (about 19.8 %) was observed between the swollen corneas in PBS (PBS-4h group) and the contracted corneas in TC (TC-4h group), and these differences were found to be statistically significant (p < 0.0001). Overall, the rate of CCT decrease was found to be lowest in TC after 4 hours.

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Fig. 3: Central corneal thickness of porcine corneas. Error bars represent standard deviation. (n=8/group).

**3.3. Quantitative microscale mapping**

Figure 4 demonstrates quantitative microscale mapping for the porcine corneas at 0, 15 and 60 mmHg. These elastic modulus maps show that corneal apex is significantly stiffer than that at para-central and peripheral regions of the corneas, p≤0.05. The inflated corneas (at 15 and 60 mmHg) exhibited an almost symmetric distribution of elastic modulus, as evident in Figs. 5b and 5c. Here, the elastic modulus was decreasing circumferentially in intervals from the centre toward the furthest point, near the limbus region. In the uninflated corneas (0 mmHg) there was an unremarkable distribution of elastic modulus as evident from Fig. 4A. Here, there was a small variation in elasticity between the centre and all of the other regions of the cornea. The elastic modulus at the centre of the uninflated corneas was 23.7±5.7 kPa and decreased by 5% at the peripheral cornea. The apex corneal elastic modulus was 228.314±10.7 kPa at 15 mmHg and decreased by 11.5% in the far peripheral region. A line of data from the nasal to the temporal region passing through the apex of inflated cornea at 15 mmHg can be represented by Equation 2 (R=0.995).

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where *x* is the location of a point on the line from the nasal to the temporal regions passing through the apex of the cornea, i.e., the point x=0 is located at the centre of the cornea.

The elastic modulus at the centre was 842.94±11.5 kPa at 60 mmHg and decreased by 15.5% in the peripheral region. The nasal-superior and nasal-inferior corners exhibit the highest elastic modulus values in all maps, which are located around 8.4 mm from the centre of the cornea, the limbus. In comparison, the temporal-superior and temporal-inferior corners have slightly lower values. As an example, Fig. 5 shows how the elastic modulus values vary from the centre of the cornea toward the nasal-superior corner at 15 mmHg.

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Fig. 4: Quantitative representation of corneal elastic modulus at varying IOPs (A) Elastic modulus map at 0 mmHg and the contour interval of 1 kPa (B) Elastic modulus map at 15 mmHg and the contour interval of 5 kPa (C) Elastic modulus map at 60 mmHg and the contour interval of 18 kPa. The scale bar represents the elastic modulus values in kPa. Each map is an average of 6 corneas.

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Fig. 5: Elastic modulus values from the centre of the cornea toward the nasal-superior corner at 15 mmHg. Errors bars represent standard deviation (n=6 corneas). The p-value refers to the statistical significance value that was measured using Mann-Whitney test.

# **Discussion**

This study aimed to measure the micromechanical properties of porcine corneas under physiological pressures using oscillatory nanoindentation. In addition, we aimed to demonstrate the potential to map the regional quantitative microscale elastic behaviour of the corneas. The main advantage of our novel method is the ability to probe localised regions of inflated corneas with a microscale resolution and map the viscoelastic properties at specific points in the cornea. We also investigated the effect of using PBS and TC, as inflation solutions, on the viscoelastic properties of the corneas with hydration time and also on corneal thickness change.

The advantages of using corneal inflation over other methods has been reported previously, where the key benefit is the ability of loading the cornea in a manner similar to in-vivo conditions (Asejczyk-Widlicka and Pierscionek, 2008; Kling and Marcos, 2013; Whitford et al., 2016). Previous studies using inflation testing have enabled monitoring of bulk geometrical changes of the inflated corneas during the loading and unloading cycles, which can then be used to predict the elasticity and hysteresis from corneal deformation (Asejczyk-Widlicka and Pierscionek, 2008; Boyce et al., 2008; Elsheikh et al., 2008, 2007; Wang et al., 2018; Whitford et al., 2016). In contrast to these methods, our method allowed the viscoelastic properties of the cornea to be precisely measured at predetermined points.

**4.1. Nanoindentation of pressurised tissue**

There are two models for inflating the corneas that have been used in biomechanics research. Firstly, the whole-eye model; where the whole eye tissue (sclera, limbus and cornea) share in the deformation that is caused by increasing the internal pressure (Asejczyk-Widlicka and Pierscionek, 2008; Kling et al., 2010; Whitford et al., 2016). Secondly, the cornea-only model (Anderson et al., 2004; Elsheikh et al., 2007; Wang et al., 2018), where the cornea is cut from the eye and fixed in a special holder for monitoring the deformation. In both inflation models, there is some complexity in calculating the biomechanical properties due to contribution or exclusion of other connected tissues to the cornea (Anderson et al., 2004) and the need for assuming some parameters. Our method overcomes some of the issues with these methods and harnesses the high spatial resolution capabilities of nanoindentation, allowing the mechanical properties of the cornea to be determined with a 100 µm indenter tip at predetermined points. Indeed, these microscale measurements with our setup primarily reflect the mechanical behaviour of the anterior lamellae of the stroma, which was shown as having a more isotropic microstructure than the deeper lamellae (Benoit et al., 2016).

Although our study is the first to provide the shear modulus for corneal tissue under pressure, we can compare our data with other studies which have provided the shear modulus for unpressured corneas. Hatami-Marbini (Hatami-Marbini, 2014) examined the effect of compressive strain on the viscoelastic behaviour of the porcine corneal stroma using torsional shear experiments. In that study, the average shear storage and loss moduli varied from 2 to 8 kPa, and 0.3 to 1.2 kPa respectively. These values are substantially lower than our values even at 0 mmHg. A number of reasons can be cited to explain the discrepancy: firstly, our data is collected at the micron level whereas Hatami-Marbini used a bulk method; secondly, our data is at 110 Hz (the resonant frequency of the nanoindenter) (Akhtar et al., 2018) whereas Hatami-Marbini’s method was at 1 Hz and thirdly, our corneas were intact and clamped whilst Hatami-Marbini used 8 mm punch samples from the central region.

There are no previous studies utilising oscillatory nanoindentation to study the cornea. However, Eberwein and colleagues utilized a depth-sensing indenter with a spherical indenter of 0.5 mm radius to calculate Young’s modulus of unpressurized (0 mmHg) human corneas by fitting the loading data to the Hertzian contact model. They reported that the elastic modulus of the central cornea was in the range of 19 kPa (Eberwein et al., 2014), which is comparable with our results for unpressurized porcine corneas (0 mmHg). Ko and his colleagues used a 5-mm-diameter cylindrical indenter to characterise elasticity of *ex vivo* inflated porcine corneas (from 12 to 40 mmHg) (Ko et al., 2013). They showed a linear relationship between elastic modulus of the corneas and IOP, ranged elastic modulus from 50 to 550 kPa at IOPs from 10 to 40 mmHg. These values are approximately in the same range of elastic modulus presented in this study. Higher values, in the range 2.7 to 3.3 MPa, were reported for rodent corneas by Wu et al. using quasi-static nanoindentation to investigate the stiffness change associated with elevated IOP (Wu et al., 2013). Another study in which AFM-based nanoindentation has been utilised to investigate the impact of hydration media on the elasticity of porcine corneas (Dias and Ziebarth, 2015). They quantified that Young’s modulus of unpressurized corneas immersed in PBS is between 130 to 299 kPa (with the intact scleral rim and without the intact scleral rim respectively) (Dias and Ziebarth, 2015). These values are slightly higher than the range of elastic modulus presented in this study. The differences in elastic modulus values can be related to the type of tip used, indentation depth, analytical solution used and the time of storage in hydration solution.

**4.2. Corneal hydration**

There are many solutions for preserving corneal tissue. Every preserving solution has its specific characteristics and can influence mechanical properties of corneas differently (Kling and Marcos, 2013; Thomasy et al., 2014). In this study, PBS and TC were used as inflating solutions for a set period of time. Hydration time is an important factor when determining mechanical properties. Hence, following some preliminary work we selected to perform 3 indents at the apex per IOP which reduced the standard deviation as compared to a larger matrix of indents where the time per test was significantly increased. We did not find any significant differences in mechanical properties between the samples in PBS-0h, TC-0h and TC-4h groups. However, this was not the case for PBS-4h. Hence, we suggest that using PBS for inflation or possibly for preservation of corneas is suitable for short periods of time e.g. 1 hour. Our findings are consistent with the finding of Dias and Ziebarth (Dias and Ziebarth, 2015) who reported that the elastic modulus increased linearly over time in corneas were immersed in PBS and swelled up for 2 hours.

CCT decreased with increasing IOP for all groups, regardless of the initial condition. The results of CCT and the trend of changing with IOP are in agreement with other studies (Dias and Ziebarth, 2015; Kling and Marcos, 2013; Vantipalli et al., 2018). Corneal swelling was most pronounced in corneas inflated by PBS. The isotonic PBS solution is commonly utilized to rinse (Rihawi et al., 2006) or preserve corneas for a short time (Dias and Ziebarth, 2015; Elsheikh et al., 2008; Vantipalli et al., 2018). However, the effect of significant tissue swelling in PBS is well-known in collagen rich tissues (Screen et al., 2006). Although PBS-incubation is associated with a reduction of mechanical properties in tendon fascicles, our data which showed an increase in stiffness with PBS matched other work on the cornea, as described in the previous section (Dias and Ziebarth, 2015). We suggest that TC is most appropriate for preserving both the mechanical properties and minimising corneal swelling, particularly if testing is required over long periods of time.

**4.3. Quantitative mapping of corneal elasticity**

The quantitative mapping data showed that the porcine corneas appeared significantly stiffer in the centre than in para-central and peripheral regions. It seems that the regional thickness of the cornea is not the main factor for determining the elasticity response, although porcine corneas are thicker in peripheral region than in the centre of the cornea (Faber et al., 2008; Sanchez et al., 2011). The inflated corneas showed a nearly symmetrical distribution of elastic modulus from the centre toward the peripheral regions and that is expected according to a model in which collagen fibrils of porcine corneas run in circumferential orientation (Hayes et al., 2007). In addition, the distribution of elastic modulus changed to be symmetric with increased IOP (from 0 to 15 mmHg), and it was slightly modified when IOP increased from 15 to 60 mmHg. A reasonable explanation could be that the stromal microstructure is modified by increased IOP, which was shown in a previous microstructural study demonstrate that the collagen lamellae slide and slightly rotate relative to each other when IOP increases(Benoit et al., 2016). Regional differences in the elastic behaviour of inflated human (Hjortdal, 1996) and porcine (Boyce et al., 2008; Whitford et al., 2016) corneas was previously proposed by utilizing digital image correlation (DIC) methods to permit three-dimensional deformation mapping. In another study in which optical coherence elastography was used to assess the effect of riboflavin UV-A crosslinking (CXL) on porcine corneal mechanical anisotropy, it was shown that the central region was stiffer than the para-central region of the CXL and control corneas (Singh et al., 2016). Our findings are in agreement with these previous studies.

The observed increase in elastic modulus in the limbal and para-limbal region can be related to the increase in the presence of elastin fibres, thickness, density and orientation of collagen fibrils to form an annulus surrounding and reinforcing the cornea. It has been reported that the aligned collagen fibrils in the limbal annulus appears to be a common feature in many animals such as in human and pig eyes (Hayes et al., 2007). The limbus of human eyes was found to exhibit a high level of x-ray scattering intensity (Newton and Meek, 1998) that was suggested due to an increase in the density and the alignment of collagen fibrils (Aghamohammadzadeh et al., 2004).

**4.4. Limitations**

The main limitation of this work is that the oscillatory nanoindentation method assumes linear viscoelastic behaviour and hence is not suitable for determining the constitutive material behaviour of the cornea. However, it does have significant advantages over other quasi-static nanoindentation methods that have been used in other studies. Another limitation was that the number of indents per cornea was reduced in order minimise testing time. Finally, there is some inaccuracy in corneal thickness values over 1100 µm, which arise due to a lack of accuracy of the pachymeter for thickness over this value. Hence, a technique such as optical coherence tomography (OCT) would be useful for thickness measurements in future work.

**4.5. Conclusions**

This study utilises oscillatory nanoindentation with inflation to determine localised measurements of viscoelastic properties of inflated corneas at the micron length scale. The effects of thickness changes and mechanical properties in different hydration solutions was also presented, with TC found to be more suitable than PBS when testing over long periods of time (4 hours). Our method has potential to be used to study localised differences between regions of healthy and diseased tissue. For example, corneal disorders such as keratoconus are characterised by a localised reduction in stiffness in specific regions.

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# **Authors Contributions**

AK conceived and planned the experiments, conducted all the experimental work, analysed the data, and edited and wrote the manuscript. BG built a MATLAB code for mapping corneal elastic properties, verified the analytical method and edited the manuscript. RA supervised all the work, conceived and planned the experiments, analysed the data and edited and wrote the manuscript.

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