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DOI: 10.14670/HH-18-029
Article type: ORIGINAL ARTICLE
Accepted: 2018-07-18
Epub ahead of print: 2018-07-18

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed. Pre-print author’s version
Ultrastructure and 3D transmission electron tomography of collagen fibrils and proteoglycans of swollen human corneal stroma

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Abstract

**Background:** The transparency of the cornea is regulated by the unique organization of collagen fibrils (CFs) which is maintained by proteoglycans (PGs). The interlacing of CF lamellae in the anterior stroma provides the biomechanical properties of the cornea.

**Objective:** To investigate the alterations of CFs and PGs in the swollen cornea, with special reference to the anterior stroma by using electron microscopy and 3D ultrastructural tomography.

**Method:** Nine healthy normal scleral corneal rings (age from 40 to 65 years) were hydrated individually in deionised water to induce swelling in the cornea. Three of them were hydrated for 2hr whereas the other three were hydrated for 48hr. The remaining three scleral normal corneal rings were used as a control. The corneas were processed for electron microscopy (EM) to study the CFs and PGs. Ultrathin sections were observed using transmission electron microscopy (JOEL 1400) and digital images of CFs, PGs and lamellae were captured using a bottom mounted Quemesa camera and iTEM Soft Imaging System. The software program ‘Composer-x64, version 3.4.2.0’ was used to construct individual 3D images from 120 digital images taken from -60 to + 60 degree angles.

**Results:** The 3D tomography showed the degeneration of microfibrils within the CFs of the swollen cornea. The CF diameter was significantly reduced and the interfibrillar spacing significantly increased in both the 2hr and 48hr hydrated corneas compared to the normal cornea. Within the hydrated corneas, the CF diameter was smaller and the interfibrillar spacing was increased in the middle and posterior stroma compared to the anterior stroma.

The PG area in both the 2hr and the 48hr hydrated cornea was reduced in the anterior stroma, whereas it was increased in middle and posterior stroma compared to the normal cornea. The density of the PGs in both the 2hr and the 48hr samples, was reduced compared to the density of PGs in the normal cornea.

**Conclusion:** The CFs, PGs and lamellae had degenerated, caused by swelling. 3D imaging demonstrated that the impairment of the microfibrils and PGs within the CF, is caused by the excessive hydration or swelling in the anterior as well as in the middle and posterior stroma. The lamellae of the anterior stroma which provides the biomechanical strength in the normal cornea, had degenerated in the swollen corneas due to the presence of the damaged CFs and PGs.
Keywords: Collagen fibrils, Proteoglycans, microfibrils, swelling, Tomography, 3D image
1. Introduction

The cornea is an outermost, curved and transparent layer of the eye, which provides the initial refraction of light on the retina. The most important part of the cornea is the stroma which is covered anteriorly by the epithelium and posteriorly by the endothelium. The stroma is composed of uniformly distributed CFs and PGs which are arranged in the parallel running lamellae. The specialized uniform organization of the CFs provides the transparency of the cornea (Maurice, 1957). The CFs are composed of collagen Type I and Type VI (Birk et al., 1988; Hahn and Birk, 1992). The endothelium plays an important role in regulating the balance in absorption of fluid in the stroma and removal of fluid out of the stroma. (Muller et al., 2011). Under high hydration conditions, the endothelial pump does not work properly and high amounts of water enter the stroma resulting in opaqueness of the cornea (Pels and Schuchard, 1985; Castoro and Bettelheim, 1988).

Hydration is an important phenomenon which keeps the CFs uniformly distributed to provide the transparency of the cornea. It is believed that hydration is regulated by the PGs and controls the uniform distribution of the CFs. The cornea contains small leucine-rich repeat PGs which are known as lumican, keratocan, mimican, decorin, byglycan and versican (Michelacci (2003). These PGs carry polysaccharide chains, the glycosaminoglycans (keratan sulphate or chondroitin sulphate). Lumicans, keratocan and mimican carry keratan sulphate chains whereas decorin, byglycan and versican carry chondroitin sulphate/dermatan sulphate chain (Iozzo, 1999).

The highly hydrophilic PGs ie (keratan sulphate) are present in the posterior stroma whereas less hydrophilic PGs (chondroitin sulphate) are present in the anterior stroma (Bettelheim and Plessy, 1975). Excessive hydration of the corneal stroma causes opacity of the cornea by disrupting the organization of the CFs and PGs (Maurice, 1957). Bettelheim and Plessy (1975) reported a gradual increase in the swelling from the anterior stroma to the posterior stroma of the hydrated cornea. The authors suggested that swelling of the cornea was related to the organization of the collagen lamellae and to the presence of different types of PGs. It has also been reported that in the posterior part keratan sulphate, a more hydrophilic PG, is prevalent, whereas in the anterior part dermatan sulphate, a much less hydrophilic PG, is prevalent (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). There are two types of PGs associated with CFs, one type of PGs were surrounding the CFs to keep them hydrated while the other type of PGs connect the CFs to each other and keep the stroma
hydrated (Hayes et al. (2017). The coated PGs around the CFs were capable of absorbing large amounts of water (Fratz and Daxer, 1993). Izzo (1999) investigated the role of PG core proteins in the assembly of CFs. The authors reported that PG core proteins bind themselves around the CFs in a way that corrugates their hydrophobic domains inside, against the CFs.

The corneal stroma is composed of collagen fibril lamellae which are interconnected to each other in the anterior stroma whereas they run parallel to the corneal surface in the posterior stroma. The anterior stroma of the human cornea is the most important and stable part of the stroma which provides the biomechanical strength and UV-B protection for the cornea (Winkler et al., 2013, Kolozsvary et al., 2002). It was predicted that the anterior stroma scattered approximately twice as much light per unit depth as the posterior stroma in humans which could be due to the differences in the anterior-posterior spatial ordering of fibrils (Freund et al., 1995). The specific organization of the lamellae and CFs, the anterior stroma of the human cornea means it is not affected by the excessive hydration (Müller et al., 2001).

The effect of swelling on the structure of the corneal stroma of sheep, rabbits, cattle and camel has been studied by various scientists (Elliot, 1980; Meek et al., 1991; Hodson et al., 1992; Doughty and Bergman, 2008; Almubrad and Akhtar, 2011). It is suggested that during swelling of the corneal stroma, water not only goes between CFs but also within the CFs (Meek et al., 1991). The mechanism of the swelling involves the endothelial pump. Under excessive hydration conditions, the endothelial pump is not able to perform its function in a normal fashion and the cornea becomes opaque (Pels and Schuchard, 1985; Castoro and Bettelheim, 1992).

Edelhauser (1989) suggested that under extreme hydration conditions, the degenerated CFs aggregate together resulting in the formation of large fibril complexes. Müller et al., (2001) studied the human corneal swelling by PBS and deionised water. The authors reported that the middle and posterior stroma of the human cornea was swollen but the anterior stroma did not swell. In the hydrated corneas the inter-fibrillar spacing increased but the diameters of the CFs was not affected.

The present study was undertaken to investigate the ultrastructural changes of the CFs and PGs of the human corneal stroma under swelling conditions, with a special emphasis on the anterior stroma. The CF diameters, interfibrillar spacing, PG area and micro-fibrillar degeneration
within the CFs were investigated by using transmission electron microscopy and 3D ultrastructural transmission electron microscopy.

2. Methods

Tissue procurement and use adhered to the tenets of the Declaration of Helsinki and local ethical regulations. The study was approved by the ethical committees in both King Saud University and King Khalid Eye Specialist Hospital (KKESH), Riyadh, Saudi Arabia.

Nine normal scleral corneal rings from healthy individuals ranging in age from 40 – 56 years were used for this study. Six of these human scleral corneal rings were hydrated individually in deionised water to induce swelling in the cornea. Three of these scleral corneal rings were hydrated for 2hr and the other three scleral corneal rings were hydrated for 48hr. The remaining three healthy scleral normal corneal rings were used as a control. The corneas were fixed in a mixture of 4% paraformaldehyde + 0.05% glutaraldehyde immediately after removal from the deionized water. The corneas were processed for electron microscopy (EM). Corneas were divided into two parts. To assess the CF diameters, the first part of each cornea was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer and post fixed in 1% osmium tetroxide then washed twice (15 x 3 min) with distilled water. The tissue was dehydrated in a graded series of ethanol (30, 50, 70, 90, and 100%) for 15 minutes in each case followed by infiltration in a mixture of ethanol: resin (1 hr) and then in 100% resin (8 x 3 hr). The samples were polymerized in Spurr resin at 70°C for 8 hours. The second part of each cornea was fixed in 2.5% glutaraldehyde containing cuprolinic blue in sodium acetate buffer and processed for electron microscopy to assess PGs (Scott and Haigh, 1985). The tissue was dehydrated in ethanol, infiltrated in resin and polymerized in resin as described above.

The ultrathin (75nm) cross sections were cut by a RMC ultra-cut microtome (Richer-Jung Ultra-cut microtome) and collected on 200 mesh copper grids. The sections were stained with 2% uranyl acetate (10minutes) and lead citrate (10minutes) then observed using transmission electron microscopy JOEL 1400 TEM (JOEL Ltd., Akishima, Japan). Digital images of CFs, PGs and lamellae were captured using a bottom mounted Quemesa camera and iT EM Soft Imaging System. Three images of the anterior, middle and posterior stroma of each sample were taken to analyze the CF diameters and PG area by using iT EM Soft Imaging System (Soft
Imaging System GmbH, Münster, Germany) analysis program. The CF diameters, interfibrillar spacing and PG area of the normal and hydrated cornea were calculated and compared using the SPSS- one-way analysis of variance (ANOVA) test.

Individual 3D images were constructed from 120 digital images taken from -60 to + 60 degree angles using the software program ‘Composer-x64, version 3.4.2.0’ (Massachusetts Institute of Technology, USA). The method for 3D imaging used here was described by Akhtar et al. (2013).

3. Results

In the anterior stroma of the normal cornea, the lamellae were thin, tightly packed and were interconnected to each other (Figure 1A) whereas in the middle and posterior stroma, the lamellae were running parallel to the corneal surface and were laid in an orthogonal arrangement. In the hydrated cornea, the lamellae in the anterior stroma were degenerated and not interconnected (Figure 1B). There were numerous electron lucent spaces within the lamellae and between the lamellae (Figure 1C). Most of the keratocytes were degenerated and contained sparse cytoplasm.

The CFs of the anterior stromal lamellae of the hydrated cornea were non-uniformly distributed and not connected by the PGs. Some of the CFs and PGs were very large whereas others were very small in the hydrated cornea (Figure 1D; Figure 1E). Within the hydrated cornea, the CFs and PGs in the middle and posterior stroma were more degenerated than the anterior stroma. In the middle and posterior stroma, the CFs were closely packed in small groups and these groups were spread wide apart by a large electron area. The organization of the CFs and PGs of the hydrated cornea were unlike the normal cornea in which the CFs were organized and connected with each other by PGs in the anterior, middle and posterior stroma (Figure 1F).

3.1 3D electron tomography

The 3D images of the normal (Figure 2B) and hydrated (Figure 2D) cornea were constructed from the 120 electron micrographs captured digitally from a bottom mounted Quemesa camera. One of the representative electron micrographs of normal and hydrated cornea is shown in
Figure 2A and Figure 2C respectively. The red and green colors were expressed by variable electron density of the particles within the CFs. In the normal cornea, the 3D images showed that the CFs were composed of the microfibrils which were intact and organized (Figure 2B). The microfibrils and PGs were organized within the CFs. In the hydrated cornea, the CFs, the microfibrils within the CFs, and PGs were degenerated and disorganized (Figure 2D).

3.2 Collagen fibril diameter

The mean diameter of CFs of the normal and the hydrated cornea was assessed by digitally color coding electron micrographs of the CFs. The electron micrographs of CFs (Figure 3A and 3C) were color coded according to their diameters (Figure 3B and 3D).

The mean CF diameters of corresponding stromal zones between normal and hydrated cornea were assessed and compared (Table 1, Figure 3). The mean diameters of CFs in the anterior (21.96±0.09nm), middle (22.35±0.13nm) and posterior stroma (19.52±0.52nm) of the 2 hr hydrated corneas were significantly (P < 0.001) smaller than the CF diameters of anterior (23.38±0.11nm), middle (24.07±0.09nm) and posterior stroma (24.19±0.08nm) of normal cornea. Within the hydrated cornea, the CF diameters of different zones were variable. Within the 2 hr hydrated corneas, the mean CF diameters of the posterior stroma (19.52±0.52nm) was significantly (P < 0.001) smaller than the mean diameters of CFs in the anterior (21.96±0.09nm) and middle (22.35±0.13nm) stroma. The CF diameters of anterior, middle and posterior stroma of the 48 hr hydrated corneas were also smaller than those of the normal cornea (Table 1). A comparison of the 2 hr hydrated corneas and 48 hr hydrated corneas, found that the CF diameters of anterior and middle stroma of 48 hr hydrated corneas were significantly smaller (p<0.0001) than those of 2 hr hydrated cornea, which was not the case for the CF diameters of the posterior stroma (Table 1).

3.3 Center-to-center interfibrillar spacing

The mean interfibrillar spacing in the anterior (39.66±0.25nm), middle (44.41±0.29nm) and posterior stroma (42.50±0.3nnm) of the 2hr hydrated cornea was significantly smaller compared to the anterior (37.02±0.18nm), middle (40.19±0.17nm) and posterior stroma (40.59±0.59nm) of the normal cornea. The interfibrillar spacing of the 48hr hydrated corneas
in all three regions was also smaller compared to the normal cornea. When comparing the 2hr and 48hr hydrated corneas, the spacing in the anterior stroma of the 48hr hydrated cornea was reduced compared to 2hr hydrated corneas whereas in the middle and posterior stroma, it was increased (Table 1).

3.4 Periphery-to-periphery interfibrillar spacing

The spacing between the CFs was measured by deducting the radius of the CFs from the intercellular spacing. The peripheral spacing of the CFs in the anterior stroma of the 2hr hydrated (34.03±0.57nm) and 48hr hydrated (35.76±0.66nm) cornea was significantly higher (P < 0.0001) compared to the normal cornea (18.75±0.30nm). The CF peripheral spacing in the 48hr hydrated cornea was significantly higher (P < 0.0001) than the 2hr hydrated cornea. The CF peripheral spacing in the middle stroma of the 2hr hydrated corneas (33.16±0.51nm) and the 48hr hydrated cornea (47.16±1.43nm) was also significantly (P < 0.0001) increased compared to the normal cornea (27.11±0.31nm). In the posterior stroma, the CF peripheral spacing of the 2hr hydrated cornea (36.37±0.63nm) and 48hr hydrated cornea (49.42±1.83nm) was also significantly (P < 0.0001) increased compared to the normal cornea (30.01±0.35nm). In both, middle and posterior stroma of the 2hr hydrated cornea was significantly increased compared to the middle and posterior stroma of the 48hr hydrated cornea (Table 1).

Within the 2hr hydrated cornea, the CF peripheral spacing of the posterior stroma was significantly higher than the anterior and middle stroma, whereas it was very similar in the anterior and middle stroma. Within the 48hr hydrated cornea, the CF peripheral spacing significantly increased from anterior to the posterior stroma.

3.5 Proteoglycans Area

The PG mean area of the normal and hydrated cornea was assessed by digital color coding according to area size. The electron micrographs of the PGs Figure 5A and 5C were color coded into digital colored images Figure 5B and 5D respectively.

The PG area in both the 2hr and 48hr hydrated corneas was reduced in the anterior stroma, whereas in the middle and posterior stroma it was significantly increased compared to the normal cornea. Within the cornea, in both the 2hr and 48hr hydrated corneas, the PGs in the
middle stroma were larger than the anterior and posterior stroma. The density of the PGs in both 2hr and 48hr samples, was reduced compared to the density of PGs in the normal cornea (Table 2). The spacing between PGs in the anterior, middle and posterior of the hydrated cornea were larger than those of the normal cornea.

4. Discussion

The present study showed a severe alteration in the organization of CFs, PGs and lamellae in the anterior, middle and posterior stroma of the hydrated cornea. The interweaving of the lamellae in the anterior was degenerated and numerous electron lucent patches were observed between the disorganized lamellae and within the lamellae. The presence of lucent spaces in the anterior stroma suggests that the water penetrated between the lamellae of the anterior stroma and disturbed the interweaving of the lamellae. Our observations were contradictory to the observations reported by Muller et al. (2011), that the middle and posterior stroma of the human cornea were swollen due to excessive hydration but that the anterior stroma was not. The authors reported that the anterior stroma (100-120µm) below the Bowman’s layer, was not affected by the extreme hydrated conditions and the interweaving of the lamellae was preserved. The authors suggested that the non-swelling of the anterior stroma could be due to close interweaving of the lamellae and CFs. Muller et al’s hypothesis was based on structural observations of the corneal stroma. They did not show the structure of the CFs and PGs and they did not analyze the CFs diameter and interfibrillar spacing of CFs statistically as in the current study.

In our study, the PG area in the hydrated cornea was reduced in the anterior stroma, whereas in the middle and posterior stroma it had increased compared to the PG area in normal cornea. The density of the PGs was reduced and the spacing between the PGs had increased. The reduction of the area size in the anterior stroma of the hydrated cornea could be due to the presence of less hydrophilic PGs (dermatan sulphate) which did not absorb water (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). It is also possible that the PGs might have dissolved in the water. The PGs of the middle and posterior stroma were mostly keratan sulphate which is highly hydrophilic and absorbs water. This results in an increase in the size of PGs. The decrease in the density of the PGs and the increase in the spacing between the PGs in the hydrated cornea could be due to the disintegration or breaking down of the PGs because
of the excessive water content in the stroma. The middle part of the stroma was less affected, maybe because it was protected by the anterior and posterior stroma.

Our observations showed that the CF diameters in the anterior, middle and posterior stroma of the swollen corneas were significantly reduced compared to those of the normal cornea. Due to high swelling conditions, the PGs surrounding the CFs (Hayes et al., 2017; Fratz and Daxer, 1993) might have washed away leaving the micro-CFs within the CF. The microfibrils within the CFs were also degenerated as a result of the high amount of water around them. We hypothesise that the reduction in the diameters of the CFs could be due to loss of PGs around the CFs and due to the degeneration of the microfibrils within the CFs. The high swelling in the stroma resulted in the degeneration of the PGs which connect the CFs resulting in large lucent spaces and large spaces between the CFs.

Within the hydrated cornea, the CF diameters in the middle and posterior stroma were significantly reduced compared to the anterior stroma. Our 3D tomography also showed the degeneration of microfibrils within the CF suggesting that water might have penetrated within the CFs resulting in the separation of the microfibrils. We believe that the microfibrils within the CF at the posterior cornea were degenerated due to the penetration of large amounts of water in the parallel running lamellae and loosely arranged CFs. This large amount of water severely damaged the highly hydrophilic PGs keratan sulphate which is attached to the core protein lumican, resulting in the degeneration of the CFs and lamellae (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). The reduction in the size of the CFs could be due to the degeneration of the lumican which regulates the CF diameters (Chakravarti et al., 2000). The diameter in the anterior stroma was also affected by water but not as much as in the posterior stroma. Despite tight interlacing of the lamellae in the anterior stroma, water still penetrated between the collagen as well as within the CFs, but not as much as in the middle and posterior stroma. In addition to that the anterior stroma contained less hydrophilic PGs (dermatan sulphate) which were less affected by the water (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). Less reduction of the CF diameters could be due to low penetration of the water in the anterior stroma and less effect of the water on the chondroitin sulphate (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). Our observations diverge from the observation of Müller et al. (2011), who reported that the CF diameter of the hydrated cornea was not different from the CF diameter of the normal cornea. The authors did not analyze the CF diameter and interfibrillar spacing statistically. Their hypothesis was based on ultrastructure observation of the corneal stroma.
The CF diameters in the anterior and middle stroma further reduced after 48hr hydration, which was not the case with the posterior stroma. This could be due to the high saturation of the posterior stroma with water, which had no more capacity to absorb the water. The maximum diameter reduction of the CFs had already occurred during the 2 hr hydration. The CF diameter was further reduced in the anterior and middle stroma after 48hr hydration, possibly due to not having been fully saturated with water within 2 hr and further penetration of water occurred during the 48hr.

Müller et al., (2001) reported that the distance between the individual CFs increased in the cornea kept in the phosphate buffer saline and deionised water. Our observation showed that the center-to-center spacing between the CFs in the hydrated cornea was increased in the anterior, middle and posterior stroma compared to the normal cornea. Within the hydrated corneas, the center-to-center spacing in the anterior stroma was significantly smaller than the center-to-center spacing in the middle and posterior stroma. This could be due to less absorption of water by the dermatan sulphate PGs and the high absorption in the middle and posterior by keratan sulphate PGs (Castoro and Bettelheim, 1988; Bettelheim and Plessy, 1975). The periphery-to-periphery spacing between CFs was larger than the center-to-center spacing. The center-to-center spacing if based on the size of the CF, could be misleading if the CF size was larger. To confirm the exact distance between the CFs and to avoid dependence of measurements of the spacing on the diameters of the CFs, we measured the distance from periphery-to-periphery.

Optimal hydration of the corneal stroma is essential to maintain the uniform distribution of the CFs which regulate the corneal transparency and this hydration is sustained by PGs (Maurice, 1967). The excessive hydration of the corneal stroma degenerated the CFs and PGs and changed their ultrastructure. The degeneration of the CFs and PGs disrupted the uniform distribution of the CF that leads to the loss of transparency. The stroma also play an important role in the biomechanical properties of the cornea. These lamellae connect to each other by numerous branches and in certain cases they insert into the Bowman’s layer (Winkler et al., 2013). The anterior stroma contained the highest degree of this interconnectivity of the transverse lamellae (Winkler et al., 2013). The transverse lamellae and their interconnectivity are essential to determine the corneal biomechanics and stress distribution, stabilize corneal shape, specify corneal curvature and prevent lamellar slippage (Komai and Ushiki 1991; Petsche et al, 2012; Winkler et al., 2013). The anterior stroma also protects the cornea from sunlight. Kolozsvari et al. (2002) have assessed the UV light
exposure on the human cornea showing that the highest absorption of the UV-B in the range from 240-400nm is by the epithelium + Bowman’s layer + 100 µm anterior stroma. The 100 µm thickness of the anterior stroma absorbed 1.8 times more UV-B than the posterior stroma. This most important part of the anterior stroma is disturbed by PRK, LASIK and LASEK surgeries. After removing the epithelium, the Bowman’s layer and the anterior stroma the laser beam remodels the cornea by burning the CFs. This remodeling causes the biomechanical weakness of the anterior stroma which may be affected by swelling of the stroma and lead to the opaqueness of the cornea.

5 Conclusion
Swelling has a degenerative impact on human corneal microfibrils within the CFs. The anterior stroma CFs become significantly smaller than those in the middle or posterior stroma, while the center-to-center and periphery-to-periphery spacing of the CFs increase due to swelling of the cornea. Meanwhile, the density of the PGs decreases, while the spacing between PGs increases in the swollen corneas, with the anterior stroma being less affected than the middle and posterior parts of the stroma. The present study demonstrates the effect of swelling on the structure and distribution of CFs, PGs and the lamellae of the cornea, in particular, its degenerative effect upon the anterior stroma, which provides biomechanical strength to the human cornea.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its Funding of this research through the Research Project no ‘RGP – VPP – 219’.
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Legends

**Figure 1**: Electron micrograph of normal and 2hr hydrated corneas; **A**) Part of the normal cornea showing interlacing of anterior stromal lamellae (Osmium tetroxide fixation); **B**) Part of the 2hr hydrated cornea showing degeneration of anterior lamellae and absence of interlacing of lamellae (Osmium tetroxide fixation); **C**) Part of the 2hr hydrated cornea showing large electron lucent spaces in the anterior stromal lamellae (Osmium tetroxide fixation); **D**) Large and small diameter CFs present in the degenerate lamellae shown in figure C (Osmium tetroxide fixation); **E**) CFs and PGs of the anterior stroma of the 2hr hydrated cornea (Cuprolinic blue fixation); **F**) CFs and PGs of the anterior stroma of the normal cornea (Cuprolinic blue fixation).

L=Lamella, V=Vacuole, CF=Collagen fibril’ LCF=Large collagen fibril, PG=Proteoglycans, MCF=Microfibrils within CF

**Figure 2**: Electron micrograph and 3D images of collagen fibrils of normal and 2hr hydrated cornea (Cuprolinic fixation); **A**) Electron micrograph of CF of anterior stroma of a normal cornea; **B**) 3D image of CF of anterior stroma of normal cornea; **C**) Electron micrograph CF of anterior stroma of 2hr hydrated cornea; **D**) 3D image of CF of anterior stroma of 2hr hydrated cornea.

CF= Collagen fibrils, PG=Proteoglycans, MCF=Microfibrils within CF

**Figure 3**: Electron micrograph and digital images collagen fibril (CF) of normal and 2hr hydrated cornea; **A**) Uniformly distributed CFs in the normal cornea in the anterior stroma (Osmium tetroxide fixation); **B**) Digital color-coded image after processing of figure A, showing large blue (20-25nm) and yellow color (26-30nm) CFs; **C**) Non-uniformly distributed CFs in the anterior stroma of 2hr hydrated cornea (Osmium tetroxide fixation); **D**) Digital color-coded image after processing of figure C, showing Red (10-15nm) and Green color (15-20nm).

**Figure 4**: Electron micrograph and digital images of the collagen fibril (CF) of anterior, middle and posterior stroma of 2hr hydrated cornea (Osmium tetroxide fixation); A) Electron micrograph of anterior stroma of the cornea; B) Digital color-coded image after processing of figure A, CF; C) Electron micrograph of middle stroma of the cornea; D) Digital color-coded image after processing of figure C; E) Electron micrograph of posterior stroma of the cornea; F) Digital color-coded image after processing of figure E.


**Figure 5**: Electron micrograph of proteoglycans (PGs) of normal and 2hr hydrated cornea; A) Electron micrograph of PGs of normal cornea; B) Digital color-coded image after processing of figure A, showing large number of big PGs; C) Electron micrograph of PGs of 2hr hydrated cornea; D) Digital color-coded image after processing of figure C, showing small number of small size PGs.

Red=30-80nm², Green= 80-130 nm², Blue=130-180 nm², Yellow=180-230 nm², Teraqata= 230-280 nm², Pink=280-330 nm².

**Figure 6** Electron micrograph of proteoglycans (PGs) of the 2hr hydrated cornea; A) Electron micrograph of PGs of the anterior stroma; B) Digital color-coded image after processing of figure A, showing small size PGs (Red); C) Electron micrograph of PGs of the middle stroma of the cornea; D) Digital color-coded image after processing of figure C, showing large size PGs (Green and Yellow); E) Electron micrograph of PGs of the posterior stroma of the cornea; F) Digital color-coded image after processing of figure D, showing slaa size PGs (Red).

Red=30-80nm², Green= 80-130 nm², Blue=130-180 nm², Yellow=180-230 nm², Aqua colour = 230-280 nm², Pink=280-330 nm².
Table 1: Mean Diameter and interfibrillar spacing of normal and hydrated corneas.

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<th>Normal Cornea</th>
<th>2 hrs. hydrated Cornea</th>
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<td><strong>Diameter ±SD (nm)</strong></td>
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<tr>
<td>N=Number of CF</td>
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<tr>
<td>Anterior</td>
<td>23.38±0.11**†, N=810</td>
<td>21.96±0.09***,†, N=918</td>
<td>19.21±0.55**,,**,†, N=1094</td>
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</tr>
<tr>
<td>Middle</td>
<td>24.07±0.09**†, N=722</td>
<td>22.35±0.13**,††, N=654</td>
<td>20.51±0.09**,††, N=594</td>
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<tr>
<td>Posterior</td>
<td>24.19±0.08*,†, N=705</td>
<td>19.52±0.52*,†,††, N=663</td>
<td>19.94±0.12,*,†,††, N=458</td>
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<tr>
<td><strong>Spacing - center to center ±SD (nm)</strong></td>
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<tr>
<td>N=Number of CF</td>
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<tr>
<td>Anterior</td>
<td>37.02±0.18*,†, N=810</td>
<td>39.66±0.25**,†, N=918</td>
<td>35.05±0.12**,,**,†, N=1094</td>
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<tr>
<td>Middle</td>
<td>40.19±0.17**,††, N=722</td>
<td>44.41±0.29*,†,††, N=663</td>
<td>46.20±0.49**,†, N=594</td>
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<tr>
<td>Posterior</td>
<td>40.59±0.59*,††,††, N=705</td>
<td>42.50±0.3*,**,†,††, N=663</td>
<td>46.92±0.74**,††, N=458</td>
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<tr>
<td><strong>Spacing - Periphery to Periphery ±SD (nm)</strong></td>
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<td>N=Number of CF</td>
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<tr>
<td>Anterior</td>
<td>18.75±0.30*,†, N=810</td>
<td>34.03±0.57*,†, N=918</td>
<td>35.76±0.66*,†, N=1094</td>
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<tr>
<td>Middle</td>
<td>27.11±0.31*,††,††, N=722</td>
<td>33.16±0.51*,**,††, N=663</td>
<td>47.16±1.43*,**,††, N=594</td>
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</tr>
<tr>
<td>Posterior</td>
<td>30.01±0.35*,††,††, N=705</td>
<td>36.37±0.63*,**,††, N=663</td>
<td>49.42±1.83*,**,††, N=458</td>
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<tr>
<td>Density/µm</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>517/µm</td>
<td>224/µm</td>
<td>244/µm</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>478/µm</td>
<td>119/µm</td>
<td>207/µm</td>
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<tr>
<td>Posterior</td>
<td>418/µm</td>
<td>161/µm</td>
<td>156/µm</td>
<td></td>
</tr>
</tbody>
</table>

N=Number of CF

* = P<0.0001 between normal and 2hr hydration, normal and 48hr hydration

**= P<0.0001 between 2hr and 48hr hydration

† = P < 0.0001 compared to anterior stroma within the cornea (Normal or 2hrs or 48hrs)

† † = P < 0.0001 compared to middle stroma within the same cornea (Normal or 2hrs or 48hrs)
Table 2: PGs Mean area of normal and hydrated Corneas.

<table>
<thead>
<tr>
<th></th>
<th>PG Area±SD (nm²)</th>
<th>PGs Density /µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior Stroma</td>
<td>83.92±1.54*,†</td>
<td>81.68±1.39*,<strong>,</strong>,†</td>
</tr>
<tr>
<td>Middle Stroma</td>
<td>79.31±1.43*,†,††</td>
<td>90.93±1.7*,<strong>,</strong>,† † †</td>
</tr>
<tr>
<td>Posterior Stroma</td>
<td>77.85±1.51*,† † †</td>
<td>82.75±2.22*,<strong>,</strong>,**,† † †</td>
</tr>
</tbody>
</table>

* = P<0.0001 between normal and 2hr hydration, normal and 48hr hydration
** = P<0.0001 between 2hr and 48hr hydration
† = P < 0.0001 compared to anterior stroma within the cornea (Normal or 2hrs or 48hrs)
† † = P < 0.0001 compared to middle stroma within the same cornea (Normal or 2hrs or 48hrs)