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# The specific architecture of the anterior stroma accounts for maintenance of corneal curvature

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## Abstract

***Aim*—To analyse the human corneal stroma in extreme hydration to discover if its structure is responsible for corneal stability.**

***Methods*—Corneas in several hydration states were used: postmortem control corneas (PM; n=3), corneas left for 1 day in phosphate buffered saline (PBS; n=4), and corneas left for 1 day (n=4), 2 days (n=4), 3 days (n=2), and 4 days (n=4) in deionised water. All corneas were fixed under standardised conditions and processed for light and electron microscopy. In addition, two fresh corneas from the operating theatre were studied which were processed 6 months after storage in sodium cacodylate buffer.**

***Results*—After 1 day in deionised water maximal stromal swelling was reached which did not change up to 4 days. The stroma of deionised water corneas (1400 µm) was much thicker than that of PBS corneas (650 µm) and PM corneas (450 µm). Deionised water treatment led to disappearance of all keratocytes leaving only remnants of nuclei and large interlamellar spaces. In these specimens the distance between the collagen fibres had increased significantly, but the diameter of the collagen fibres did not seem to be affected. A remarkable observation was that the most anterior part of the stroma (100–120 µm) in all deionised water specimens and those stored for 6 months in buffer was not swollen, indicating that the tightly interwoven anterior lamellae are resistant to extreme non-physiological hydration states.**

***Conclusions*—The rigidity of the most anterior part of the corneal stroma in extreme hydration states points to an important role in maintenance of corneal curvature. Since a large part of this rigid anterior part of the stroma is either removed (PRK) or intersected (LASIK), it is possible that in the long run patients who underwent refractive surgery may be confronted with optical problems.**

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A high refractive index and a stable rotation symmetric curvature are essential for optimal refractive power and minimal astigmatism of the cornea. In cases of disease, especially of the endothelium, the corneal stroma tends to swell and become opaque. It is known that swelling mainly occurs in the posterior-anterior direction and is lowest in the most anterior part.[1] This increase in thickness will cause opaqueness due to increased light scatter, decrease in overall refractive index, and may also give rise to changes in curvature.[2–4] The differential swelling properties of anterior and posterior stroma are often ascribed to an osmotic diffusion gradient caused by differences in proteoglycans.[1 5 6] However, stability of the anterior stroma could also account for the close to optimal refraction in cases of mild disturbance. Moreover, it was also noticed that even extreme stromal swelling hardly affects corneal curvature. The stability of the anterior surface was ascribed to a preferred orientation of collagen lamellae.[7 8] A preferred orientation was indeed observed in histological studies.[9 10] Contrary to the orthogonally arranged lamellae in the mid and posterior stroma, the collagen bundles in the anterior stroma are undulating and interwoven.[11–13] Such differences in organisation may cause different cohesive strengths between collagen bundles in the anterior and posterior stroma and may account, for instance, for the easy separation of the collagen lamellae in the posterior stroma.[14] On the basis of the differential density of the collagen fibres in humans it was predicted that at any given wavelength approximately twice as much light per unit depth will scatter in the anterior stroma compared with the posterior stroma.[15] This prediction is supported by refractive index measurements showing a decrease from 1.401 at the anterior surface (epithelium) via 1.380 (stroma) to 1.373 at the posterior surface.[16]

It is well known that in both types of refractive surgery—namely, photorefractive keratectomy (PRK),[17–19] and laser in situ keratomileusis (LASIK),[20–23] the anterior part of the stroma is removed. With PRK the central epithelium, Bowman's layer, and the anterior stroma are ablated whereas with LASIK epithelium and Bowman's layer and part of the anterior stroma remain within the flap and finally re-cover the ablated zone. Most patients gain a well corrected visual acuity but in some cases haze is observed. Despite numerous follow up studies on the cause of haze[18 24–27] and refractive errors[26 28] no attention has been paid to long term effects of refractive surgery on surface curvature and refractive index. This is surprising because it has already been suggested in the early 1990s, on basis of the difference in cohesive strength between superior/inferior periphery and nasal/ temporal periphery, that refractive surgery might influence the rotation symmetry of the cornea[8] and thus cause astigmatism.

The present study aimed to analyse the architecture and the swelling properties of the cornea in relation to the stability of the anterior stroma. To realise this, the osmotic factor is almost completely eliminated by immersion of human corneas in pure deionised water.

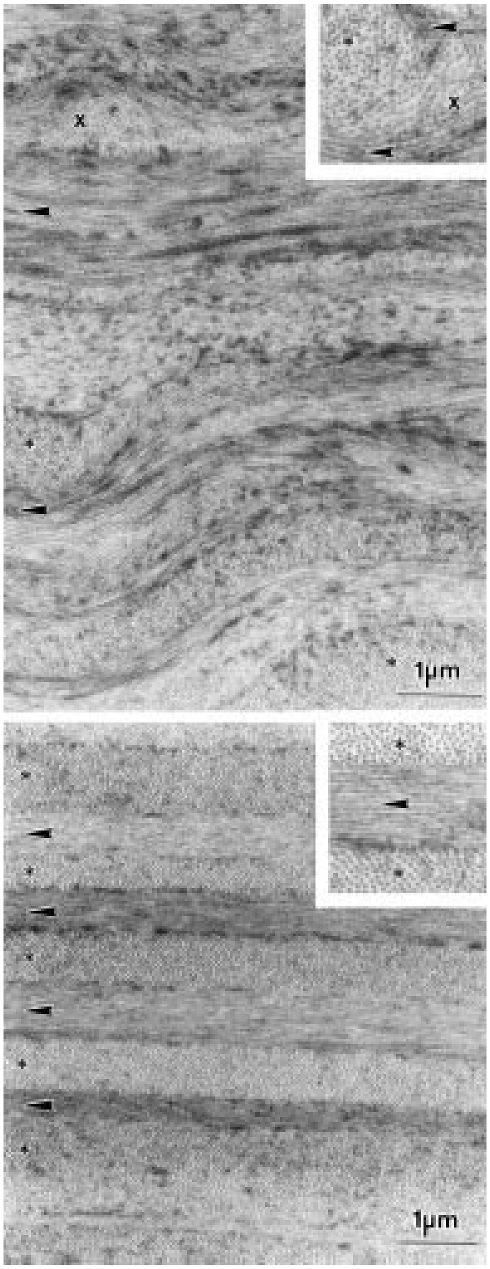

*Figure 1 (Top) Electron micrograph of a cross section through the anterior stroma of the human cornea. Collagen lamellae are alternated and undulated. The different collagen bundles cross sectioned (*), longitudinally sectioned (arrowhead), and obliquely sectioned (x) alternate at irregular distances. (Bottom) Electron micrograph of the posterior stroma shows strictly alternating cross sectioned (*) and longitudinal sectioned (arrowhead) collagen bundles at more or less regular distances.*

## Materials and methods

In the present study, 25 corneas from 25 subjects were evaluated. Corneas discarded for transplantation on the basis of endothelial cell density were obtained from the Cornea Bank Amsterdam. Methods for securing human tissue were humane, and the tenets of the Declaration of Helsinki were followed.

Whole postmortem corneas (corneoscleral buttons) (PM; n=3), corneas left for 1 day in phosphate buffered saline (PBS; n=4), and corneas left for 1 day (n=4), 2 days (n=4), 3 days (n=2), or 4 days (n=4) in deionised water were fixed overnight at 4°C in 1.25% glutaraldehyde/1% paraformaldehyde in 0.08 M cacodylate buffer (pH 7.4). Two corneas stored for more than 6 months in cacodylate buffer were also fixed. After postfixation in 1% osmium tetroxide supplemented with 1.5% ferrocyanide in 0.1 M sodium cacodylate buffer (pH7.4) pieces were dehydrated in a graded series of ethanols and embedded in epoxy resin. Fresh corneas from enucleated eyes (melanoma) collected in a previous study[29 30] and processed in an identical way were used for comparison with those of the treated groups. Semithin cross sections were stained with toluidine blue and photographed in a Univar light microscope (Reichert, Vienna, Austria). From all groups, three sections per specimen were made and drawn with a drawing tube attached to a light microscope. Since Descemet folds in swollen corneas cause great variations in stromal thickness the mean values at several points in the sections were measured with a calibrated object micrometer and the mean thickness was calculated. Also the thickness of the most anterior non-swollen stroma defined as the part without extracellular spaces was measured in this way. Ultrathin sections stained with uranyl acetate and lead citrate were inspected in Philips EM 201 and CM 12 electron microscopes (Philips Industries, Eindhoven, Netherlands).

Measurements were tested with a one way analysis of variance. Differences were considered significant when $p < 5\%$.

## Results

In cross sections of control corneas the differences between alternating collagen lamellae in the anterior and posterior stroma are very clear. The anterior stroma is characterised by few straight and many undulating collagen bundles and the collagen fibres are not parallel aligned in longitudinal sectioned bundles. Between the collagen fibres many randomly dispersed electron dense spots of amorphous extracellular matrix are present (Fig 1(top)). In contrast, amorphous matrix is less abundant in the posterior stroma and largely restricted to the borderlines of the collagen bundles. The orthogonally aligned collagen bundles are almost equal in thickness and consist of parallel running collagen fibres (Fig 1(bottom)).

Cross sections of corneas after various treatments show great differences in thickness as shown in Figure 2. The epithelial lining in postmortem and PBS corneas is smooth whereas in water stored specimens it is irregular because of degenerated cells. In PM control corneas with a stromal thickness of 450 µm, keratocytes and collagen lamellae are regularly aligned (Fig 2A). One day in PBS leads to undulations in Descemet's membrane and the corneal thickness is approximately 650 µm. Although keratocyte profiles in the posterior stroma of these specimens are shorter and thicker, no spaces between collagen lamellae were observed (Fig 2B). Radical changes occur when corneas are stored in water in which a stromal thickness of up to 1200 µm was reached. Keratocytes have, independent of the time of storage, lost their original long slender morphology and few irregularly shaped nuclei with a thin rim of cytoplasm are left (Fig 2C and D). The remnants of the keratocytes are

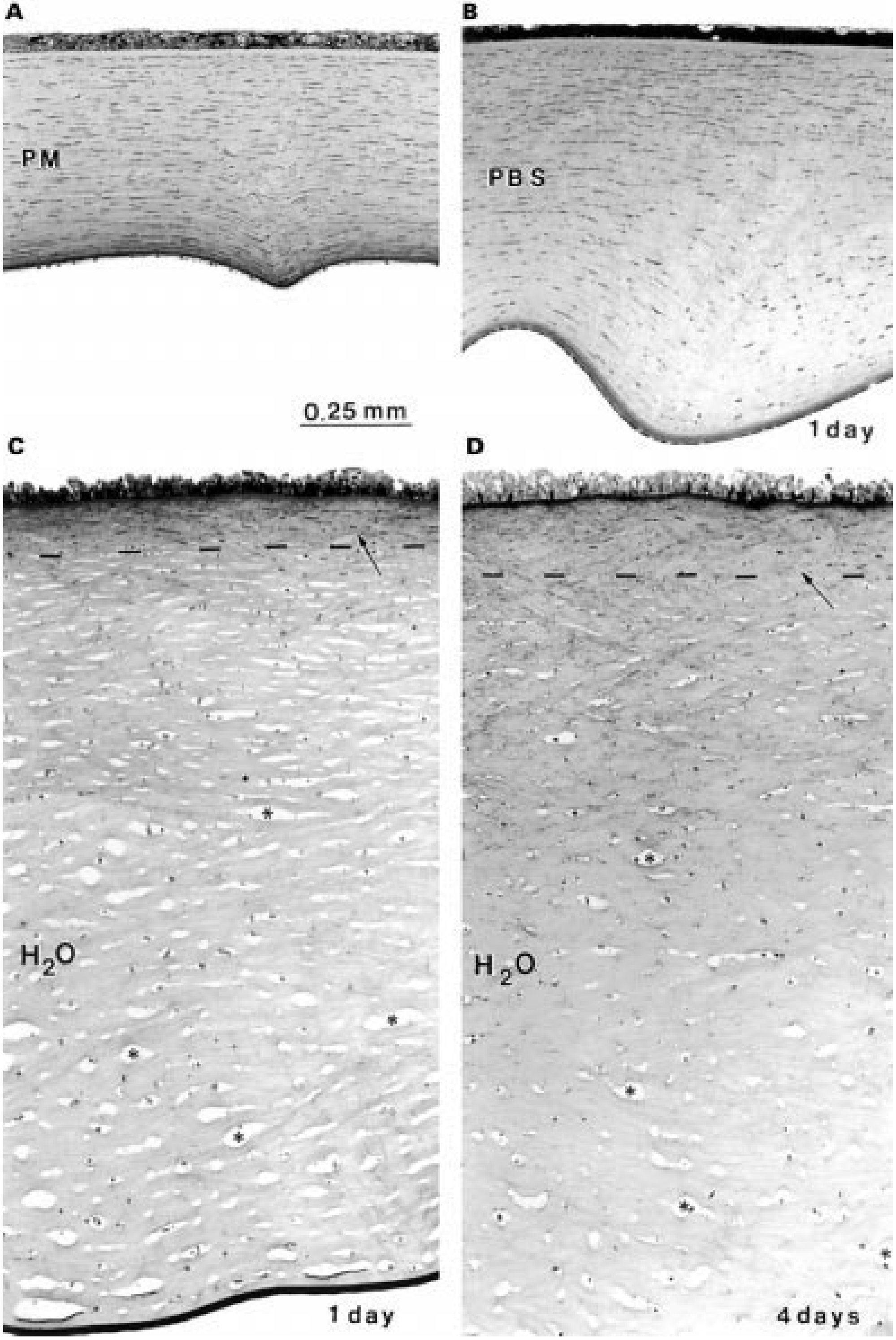

*Figure 2 Light micrographs of semithin sections from corneas at different states of hydration. (A) Post mortem (PM); (B) phosphate buffered saline (PBS); (C) 1 day deionised water, and (D) 4 days deionised water. The magnification for all micrographs is the same. The most anterior stroma, above the broken line, is not affected by hydration (C and D, arrows). Most keratocytes in the mid and posterior stroma are shrunken or have disappeared and large interlaminar spaces (*) are left.*

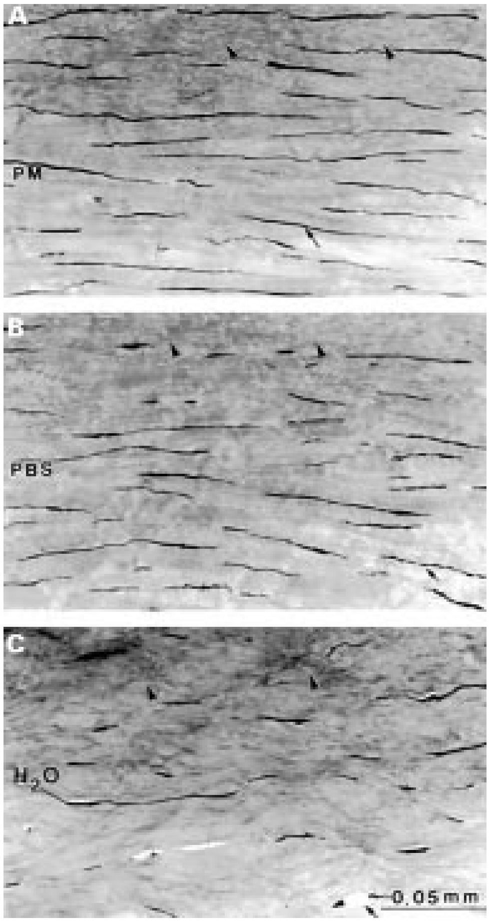

*Figure 3 Light micrographs of the most anterior stroma in (A) post mortem (PM); (B) phosphate buffered saline (PBS), and (C) deionised water ($H_2O$). In (A) and (B) more keratocyte profiles are present (arrows) than in (C). The undulated collagen bundles (arrowheads) in (A) and (B) are similar; (C) is different and characterised by irregularly arranged grey structures.*

surrounded by large extracellular spaces devoid of electron dense matrix. Nevertheless, in these specimens wavy collagen lamellae can still be discerned.

A most surprising observation is that 100–120 μm below Bowman's layer (broken lines in Fig 2C and D) the stroma is relatively unaffected under these extreme swelling conditions. This phenomenon is also observed in corneas stored for 6 months in buffer (not shown).

At higher magnifications there is hardly any difference between PM (Fig 3A) and PBS corneas (Fig 3B) in the most anterior part of the stroma and only slight differences were found between the stroma of the previous two and that of deionised water treated corneas (Fig 3C). In deionised water treated corneas fewer keratocyte profiles are present and the amorphous extracellular matrix is less homogeneously distributed (Fig 3C). In the same figure a few remnants of degenerated keratocytes are found.

At the ultrastructural level greater differences were expected. However, electron microscopic images do not show significant differences in diameter of the collagen fibres between control corneas and deionised water corneas. Only the distance between the individual fibres increases: compare PM (Fig 4A), PBS (Fig 4B), and water treated (Fig 4E and F) corneas. Images are shown in the longitudinal direction because cross sectioned collagen fibres in the posterior stroma of water corneas are difficult to visualise because of poor contrast.

The irregular amorphous matrix seen in the most anterior 100–120 μm of the stroma in Figure 3C, is more evident in electron micrographs, especially after 1 day in water (Fig 4C). This figure also illustrates the pattern of interwoven collagen fibres. As mentioned before, most of the amorphous material and most keratocytes in the posterior stroma have disappeared and few indented nuclei surrounded by a thin rim of cytoplasm are left (Fig 4D).

In light micrographs, the electron dense extracellular matrix in mid and posterior stroma of deionised water treated corneas seems to have vanished (Fig 2C and D). Nevertheless, at 1 day (Fig 4E) and at 4 days (Fig 4F) fine irregularly dispersed material between the collagen fibres is found which points at a redistribution of the matrix.

Figure 5, summarising the measurements on stromal thickness, shows that maximal swelling is reached after 1 day of deionised water treatment. There is no significant difference in mean stromal thickness between the groups after 1, 2, 3, and 4 days in deionised water. The stroma of all these corneas is significantly thicker than that in PBS and PM corneas. The standard deviation for the non-swollen anterior part of the stroma is negligible compared with the swollen mid-posterior part with an outlier at 1 day.

## Discussion

Maintenance of a rotation-symmetric curvature and high refractive index at the interface between air and the anterior surface of the cornea is of ultimate importance for the refractive power of the cornea and its stigmatic properties. The current study suggests that the specific architecture of the most anterior part (100–120 μm) of the stroma prevents changes in the morphology of this stromal region even after extreme swelling of up to two to three times its original thickness.

It is well known that the stroma is swollen in organ cultured corneas. However, the smooth curvature at anterior side is not affected under these conditions. As the diameter of the cornea hardly changes swelling is compensated by large undulations in Descemet's membrane at the posterior side.[9 31 32] The difference in swelling behaviour between anterior and posterior stroma is further evidenced by the fact that isolated stromal tissue from rabbit and humans shows negligible extensibility under stress, whereas isolated Descemet's membrane in both species is very extensible under similar conditions.[33]

Many studies that focused on corneal swelling behaviour noticed a gradual decrease in swelling from the posterior to the anterior side.[1 5 34 35] This process was thought to be

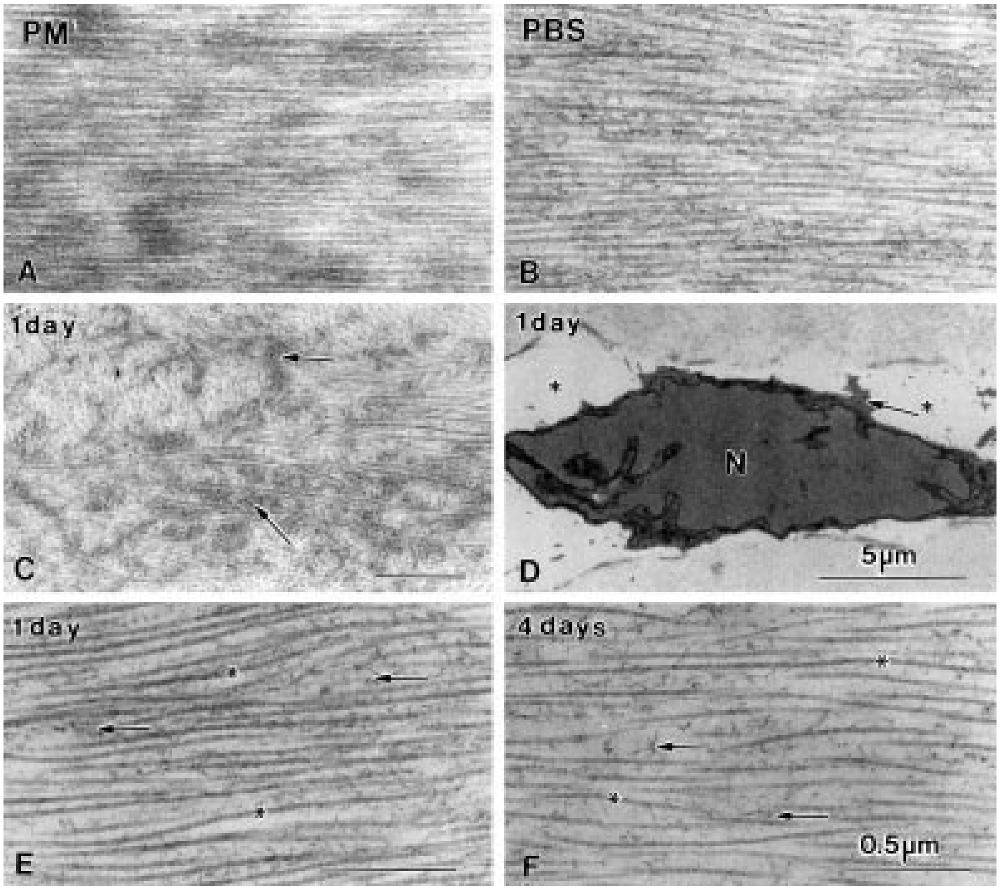

*Figure 4 Longitudinal collagen fibres of a PM cornea (A) and a PBS cornea (B). The irregular grey structures in Figure 3C after 1 day in deionised water represent amorphous extracellular matrix (C, arrows). Only occasionally keratocyte nuclei with a small rim of cytoplasm are observed in mid stroma. Treatment with deionised water does not allow well stained cross sectioned collagen fibres in the posterior stroma. Longitudinal collagen fibres (*) surrounded by amorphous matrix (arrow) can be visualised (E and F). All bars represent 0.5 µm.*

related to the organisation of the collagen lamellae and to the presence of different types of proteoglycans. In the posterior part keratan sulphate, a more hydrophilic proteoglycan, is prevalent, whereas in the anterior part dermatan sulphate, a much less hydrophylic proteoglycan, is prevalent.[5 34] Furthermore, it was suggested that proteoglycans could be lost during organ culture.[6] However, swelling does not seem to induce a loss but a rearrangement of proteoglycans because a reduction of less than 1% proteoglycans was found after organ culture for 28 days.[36] Although collagen fibres in the posterior stroma of deionised water treated specimens are surrounded by faintly stained structures the amorphous dense matrix as seen in control corneas was absent at the ultrastructural level.

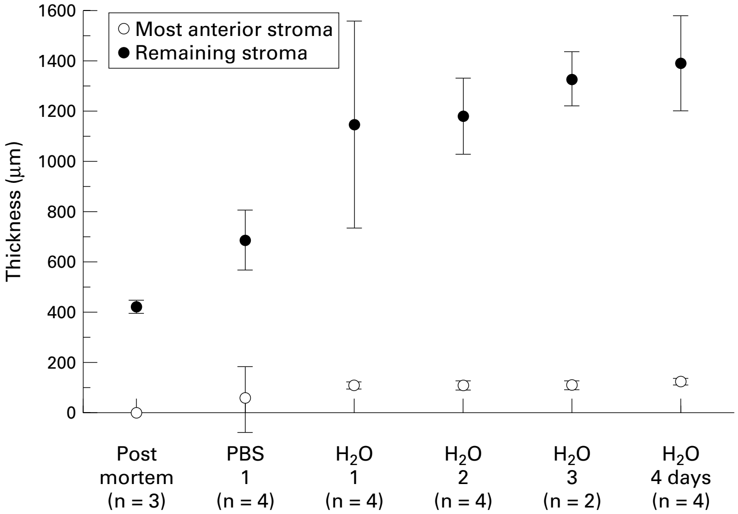

*Figure 5 Thickness of the corneal stroma after treatment with deionised water for 1, 2, 3 and 4 days. These groups are compared with PM and PBS treated tissue.*

Recently, Maurice[37] showed that very thin sections of the stroma do not fall apart after immersion in water indicating that a structural framework holds the collagen fibres together. The current study supports this observation that collagen lamellae remain intact albeit with larger interfibrillary distances, whereas keratocytes are severely affected. When these keratocytes are compared with those in PBS and organ cultured corneas, where the osmotic factor is not completely eliminated, the cells show much less deterioration in the latter cases.

Under extreme hydration states the endothelial pumps are not able to function properly. As a consequence the balance between fluid absorption and exclusion by the endothelium is disturbed. Such corneas dramatically absorb fluid and become completely opaque (personal observation).[32 34] A decrease in corneal clearness might also occur at a later age in humans when the endothelial pump capacity has been shown to decrease.[38]

In a later study Hodson[39] suggested that when corneas swell, the charge separation of the extracellular matrix increases and the forces to initiate swelling decrease. A decrease in swelling forces or imbibition pressure was already described in the early 1970s.[40] It was thought that a decrease in imbibition pressure with time was due to leaking of polysaccharides. A significant loss of proteoglycans has been disproved[36] but the loss of polysaccharides was not measured. Here, the important function of the endothelium to maintain the stroma at physiological conditions by preventing fluid from entering the corneal stroma is emphasised. Fluid was thought to enter the cornea from both the endothelial and epithelial side. However, entrance at the epithelial side was negligible because the stroma only became very thick when the endothelium was removed.[35] A cornea without endothelium[35] swells even more than an intact cornea in deionised water, as described in the current study.

The entrance of fluid at the endothelial side and the organisation of the collagen lamellae in the posterior stroma seem to be closely related to the gradual decrease in swelling from the posterior to the anterior side. Lamellae in the posterior stroma are strictly orthogonally aligned and from mid stroma on they gradually become interwoven[11] to end in the formation of a cohesive structure in the most anterior 100–120 µm. Previous ultrastructural studies[9 10 12 13] corroborate these observations.

In an attempt to count the number of lamellae in semithin sections of epoxy resin embedded tissue, the stroma was divided into three parts—0.4 for the posterior, 0.4 for mid, and 0.2 for the anterior part.[10] Quantification of the number of lamellae in the anterior part was almost impossible and remarkably this coincides with the unaffected stroma in the deionised water treated specimens. Numerous observations on morphological and biophysical properties explain why the cornea maintains a correct curvature after removal of the endothelium[35] and after organ culture without addition of dextran to the medium.[32] These data fit perfectly well with the decrease in refractive index from anterior to posterior surface[16] and the prediction that the most anterior stroma will scatter approximately twice the amount of light in comparison with mid and posterior stroma.[15]

A cut in the anterior stroma of rabbit corneas leads to a retraction of collagen lamellae resulting in a wavy appearance with large amplitudes.[3] Such a cut was not made in human corneas. However, after making an incision parallel to the collagen bundles at the limbal side, fresh human corneas can be separated into a posterior and an anterior part without showing the undulations (personal observations) as found in the case of rabbits. Also, in severe hydration states rabbit corneas differ from those of humans in forming large collagen-free spaces described as lakes.[41] Such lakes were neither observed in oedematous organ cultured corneas nor in deionised water corneas, indicating that rabbit and human corneas are indeed different.

Edelhauser[6] suggested that extreme hydration might result in aggregation of collagen fibres to form large fibre complexes. This idea can be ruled out because fibres in water and in buffer for 6 months have the same diameter as those observed in control corneas. Only the distance between collagen fibres becomes extremely large in the posterior stroma. Other authors[42] suggested a coating of collagen fibres by proteoglycans which are capable of absorbing large quantities of water. Depending on the degree of hydration the diameter of the coating was thought to vary between 36, 5, and 80 nm. Scott[43] proposed a similar arrangement with comparable diameters of the coating. Collagen fibres in a complete non-physiological hydrated state have certainly lost their coating because distances over 500 nm were found.

Refractive surgery was introduced to correct refractive errors in myopic patients. LASIK[20–22] leaves epithelium and Bowman's layer intact because a flap is made before laser treatment. PRK[17–19] removes the epithelium, Bowman's layer, and part of the anterior stroma.

Despite refractive regression in some cases in the first years,[19 44 45] follow up studies show that PRK is safe for up to 6 years.[44–46] However, the question remains as to what the long lasting effects in terms of decades of such treatments on the stability and the swelling properties of the cornea will be.

PRK and LASIK aim at the same results but the approaches are different. In the case of LASIK 160–180 µm thick flaps or discs are introduced.[21–23] Since epithelium and Bowman's layer are together approximately 60 µm, the remaining part of the flap is 100–120 µm anterior stroma. This appears to be exactly the thickness of the unaffected anterior stroma. It might be that the complex uppermost 120 µm does not allow a smooth microkeratome cut so that refractive surgeons are not able to make thinner flaps with LASIK. LASIK studies do not report flaps of less than 160 µm,[21–23] which means that the flap always includes the most stable part of the cornea and is connected at the temporal side of the cornea by a small hinge. In a histological study in humans in which LASIK was performed before enucleation of one eye it was demonstrated that mainly fibronectin and tenascin were formed at the border between flap and stroma.[21] These adhesive glycoproteins are present between epithelium and Bowman's layer and can easily be disrupted to perform additional laser treatments. There is no need to wonder why various patients suffer from wrinkles within the flap[47] and epithelial ingrowth.[21] At present the percentage of such complications is less than 5%[23] but there are additional changes in corneal topography.[48]

PRK is different from LASIK because a great part of the most anterior stroma is ablated. Bowman's layer does not recover and irregularities on the surface are compensated by ingrowth of epithelial cells (personal observation). Interference with the most tightly interwoven part of the cornea may result in visual problems. A significant proportion of treated patients may show refractive regression, haze, or astigmatism after both types of treatment.[18 22 23 28 49 50] Astigmatism is a clinical complication related to irregularities in the corneal curvature. Formation of these irregularities may be due to a reduction in cohesiveness of the collagen bundles in the central corneal stroma. Our results indicate that the most rigid part of the stroma is ablated (PRK) or intersected (LASIK), thus weakening the stability of the cornea. This emphasises that people who underwent refractive surgery may have an increased risk of optical problems.

## Conclusions

The specific architecture of the most (120 µm) anterior part of the corneal stroma is responsible for its stability, even under extreme swelling conditions.

This stability explains the maintenance of corneal curvature after long term storage.

Since a large part of this rigid anterior part of the stroma is either removed (PRK) or intersected (LASIK), it is possible that in the long run patients who underwent refractive surgery may be confronted with optical problems.

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