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Interlamellar cohesion after corneal crosslinking using riboflavin and ultraviolet A light

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ABSTRACT

Aims Collagen crosslinking treatment of progressive keratoconus using the photosensitiser riboflavin and ultraviolet A light of 370 nm wavelength has been shown to increase significantly the tensile strength of corneal collagen by about 300%. In keratoconus, interlamellar and interfibrillar slippage have been proposed as pathogenetic mechanisms. Therefore, the aim of this study was to assess the impact of collagen crosslinking on the interlamellar cohesive force.

Methods 72 post mortem porcine eyes were divided into six different treatment groups: the untreated control group, the standard crosslinking group, the hypo-osmolar crosslinking group, the stromal swelling group, the formaldehyde group and the α-amylase group. An anterior 9×4 mm strip of 400 μm thickness was prepared using a lamellar rotating microkeratome. For interlamellar cohesive force measurements a splitting plane was created at 50% depth. Force–distance profiles were recorded using a microcomputer-controlled biomaterial testing machine.

Results The mean interlamellar cohesive force was 0.24 N/mm in the untreated control group, 0.26 N/mm in the standard crosslinking group, 0.25 N/mm in the hypo-osmolar crosslinking group, 0.23 N/mm in hydrated corneas, 0.27 N/mm in the formaldehyde group without statistically significant difference. Only the values of the α-amylase group were statistically significantly lowered by 31.5% to 0.16 N/mm.

Conclusions Surprisingly, corneal crosslinking does not increase the interlamellar cohesive force. In the α-amylase group the cohesive force was mainly decreased because of the digestion of proteoglycans. Crosslinking seems to stabilise only inter- and intrafibrillar, but not interlamellar cohesion.

INTRODUCTION

Corneal crosslinking treatment of progressive keratoconus using the photosensitiser riboflavin and ultraviolet A light (UVA) was introduced by Wollensak *et al*[1] in Germany in 2003 and has become increasingly popular in recent years.[2 3] Long-term results have confirmed the earlier positive results.[3]

The success of the new crosslinking method in the treatment of progressive keratoconus is based primarily on its biomechanical stiffening effect, stabilising the corneal collagen fibril network and halting the progression of ectasia ('freezing').[2 4 5] The crosslinking effect is strongest in the anterior stroma as found in numerous biomechanical,[6] histological[2 7] and hydration studies.[8]

Ultrastructurally, the collagen fibre diameter is increased by 12.2% in the anterior stroma after crosslinking concurrent with intrafibrillar collagen crosslinks,[7] and there is almost complete absence of hydration effects in the anterior crosslinked zone compatible with interfibrillar collagen crosslinks.[8] Recent studies have suggested that in keratoconus a reduction in the stromal cohesion may lead to interfibrillar or interlamellar slippage of collagen fibres.[9–12]

Therefore, in the present study we tried to investigate the possible changes of interlamellar cohesion after crosslinking to better understand the way crosslinking functions in the treatment of progressive keratoconus.

MATERIALS AND METHODS

Sample preparation

Seventy-two porcine eyes were retrieved from the local abattoir within 24 h post mortem. The horizontal meridian was identified by the elliptical shape of the cornea and marked with a marker pen. Only clear corneas were used. The epithelium was carefully removed. Anterior circular flaps of 400 μm thickness and 9 mm diameter were cut using a Draeger lamellar rotating microkeratome (Storz Instrument GmbH, Heidelberg, Germany). Finally, a 4×9 mm rectangular central strip was cut along the horizontal meridian of the circular lamellar flap.

Treatment groups

The 72 porcine eyes were divided into six different groups with twelve samples per group:

1. The untreated control group.
2. The standard crosslinking group, in which the porcine eyes were crosslinked according to the standard protocol of Wollensak *et al*[1] with removal of the epithelium and subsequent application of the standard riboflavin solution (1 mg riboflavin 5-phosphate in 1 ml 20% dextran T-500 dissolved in physiological saline solution) for 5 min before the irradiation, and every 5 min during the 30 min of irradiation using a commercial UVA diode system (Peschke Meditrade GmbH, Nuremburg, Germany) with a surface irradiance of 3 mW/cm^2 and a focusing distance of 5 cm.
3. The hypo-osmolar crosslinking group, which was treated like group 2 except for applying a hypo-osmolar (310 mOsmol/l) riboflavin solution without dextran, as is used sometimes in thin keratoconus corneas inducing moderate stromal swelling.
4. The group in which the samples were placed into a moist chamber with physiological saline for 24 h inducing stromal swelling alone (without crosslinking).
5. The formaldehyde group with maximum crosslinking, with the samples placed into a 3.5% neutral buffered formaldehyde solution for 48 h.

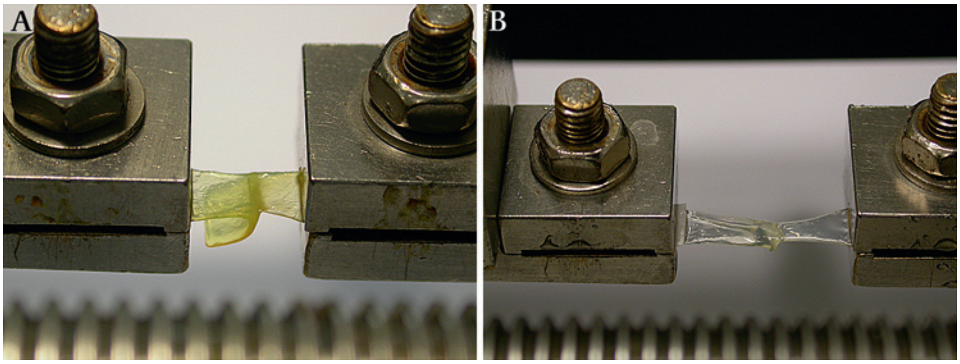

Figure 1 Cohesive force measurements of (A) riboflavin/ultraviolet A light crosslinked and (B) α-amylase-treated anterior porcine corneal flaps being torn apart at 200 μm depth by the clamps of a biomaterial testing machine. Note the yellowish tint of the crosslinked samples due to the photosensitiser riboflavin and the increased transparency of the α-amylase-treated sample.

6. The α-amylase group, in which the corneas were kept in an aqueous solution of α-amylase (from *Aspergillus oryzae*, ≥800 FAU/g, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 72 h at room temperature to digest the proteoglycans.

Pachymetry

After treatment, the thickness of the initially 400 μm thin flaps was determined using ultrasound pachymetry (Pachette, Technomed, Baesweiler, Germany).

Interlamellar cohesive force measurements

Anterior corneal (4×9 mm) strips were incised and carefully cleaved at 50% depth for a 2 mm length on one end of the strip. Once the splitting plane had been created, the anterior and posterior end were fixed in the two clamps of a microcomputer-controlled biomaterial testing machine (MINIMAT; Rheometric Scientific GmbH, Bensheim, Germany; figure 1A,B). After an initial build-up of tension, the stroma split up at a constant rate of 3 mm/min and the cohesive force was measured until the strips were split for a distance of about 7 mm. Force—distance profiles were recorded and the average force or load determined. As cohesive strength is defined as the force in N needed to split a stromal sample of 1 mm width along a plane parallel to the surface, the load values obtained for 4 mm wide strips were divided by 4 (figure 2A,B, table 1). For statistical analysis, the p values of the biomechanical data were calculated using a Student t test for the comparison with the control group.

Histology

Characteristic samples from each treatment group were fixed in 4% formaldehyde for 3 days and embedded in paraffin. Paraffin cross-sections (4 μm) were cut and stained with haematoxylin—eosin and periodic acid—Schiff.

RESULTS

Gross appearance and pachymetry

The flaps in all treatment groups except for groups 3 and 4 with oedema appeared to be transparent. The swelling effect in groups 3 and 4 was rather weak because only the anterior 400 μm of the stroma was used, which is rather resistant to swelling. After swelling, the mean thickness of the hypo-osmolar group 3 was increased to 442±23 μm and of the physiological saline group 4 to 433±27 μm. The pachymetry readings for the control group were 402±18 μm, the crosslinking group 398±12 μm, the formaldehyde group 395±7 μm and the α-amylase group 376±22 μm. The samples from groups 2 and 3 had a yellowish tint due to the applied riboflavin solution (figure 1A). The α-amylase-treated samples appeared more transparent than the flaps of all the other groups, including the controls (figure 1B).

Biomechanical cohesive force measurements (table 1)

After an initial build-up of tension, the stroma split up with a rather uniform force.

Characteristic force (load)—extension profiles with localised fluctuations in required tearing force (figure 2A,B) were registered.[13 14] Only the values of the α-amylase group were statistically significantly different from the control group, with a decrease in cohesive force of 31.5% ($p<0.0001$; table 1). The slight increase in interlamellar cohesive force after crosslinking by 5.7% was not statistically significant ($p<0.31$). The number of samples required to exclude a type II error for the crosslinking changes would be 60 samples for both the crosslinking and control group, demonstrating a low probability of a false-negative result.

Histology

Torn lamellae could be seen at the interface of the tearing plane in all cases. In the control group (figure 3A), and even more so in the crosslinking group, the tissue appeared more compacted

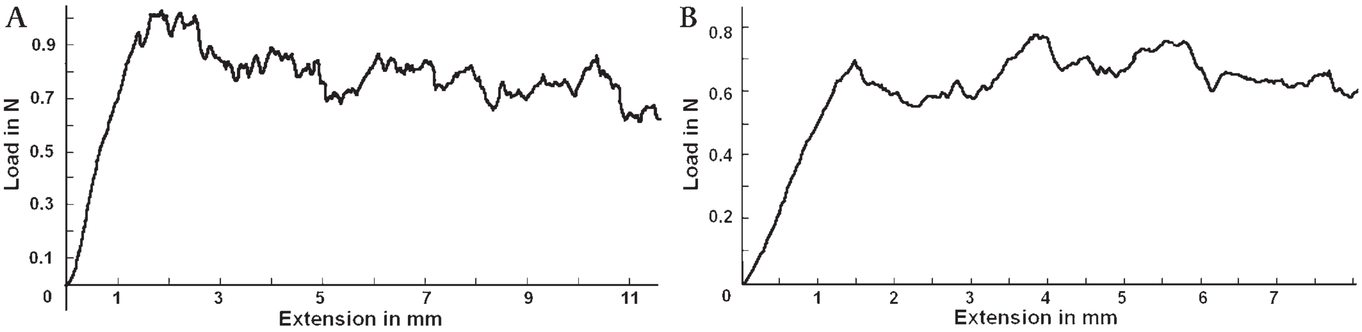

Figure 2 Representative load-extension profiles of (A) riboflavin/ultraviolet A light crosslinked and (B) α-amylase-treated specimens. Note the characteristic spikes corresponding to interlamellar tears and the significantly reduced tearing load in the α-amylase-treated specimens.

Table 1 Overview of the cohesive force measurements

Sample group	Controls	Crosslinking	Crosslinking with swelling	24 h swelling only	48 h formaldehyde	72 h α-amylase
Cohesive force (N/mm) (mean±SD)	0.24±0.025	0.256±0.032	0.253±0.049	0.232±0.024	0.267±0.034	0.166±0.034
Change (%)	–	+5.7	+4.5	−4.4	+10	−31.5
p Value	–	<0.310 (NS)	<0.067 (NS)	<0.382 (NS)	<0.067 (NS)	<0.0001*

*Significant difference.

(figure 3B) and the lamellae straightened; in the oedematous groups 3 and 4 only a moderate degree of stromal oedema was seen; in the α-amylase group the collagen lamellae appeared rather wavy, there was some oedema and the cell nuclei appeared less basophilic (figure 3C).

DISCUSSION

This study has shown that collagen crosslinking using riboflavin and UVA or even the strong crosslinker formaldehyde does not have a significant effect on interlamellar cohesion in the anterior stroma. This finding is very surprising. It appears that collagen crosslinks are mainly intra- and interfibrillar, but not interlamellar. On the other hand, the digestion of proteoglycans by α-amylase led to a significant decrease of interlamellar cohesion by 31.5% demonstrating the important role of the proteoglycan–glycosaminoglycan complexes for interlamellar stabilisation.

The lamellar structure of the cornea is rather complex and not yet fully elucidated. The simplified lamellar concept is that the cornea consists of 300–500 smooth lamellar layers, similar to a stack of 300 sheets of paper, that can slide over each other when subjected to shear. Detailed studies using scanning electron microscopy,[15–17] microdissection analysis,[15 16] transmission electron microscopy,[18] polarised light microscopy or x-ray diffraction[19] have demonstrated that the cornea resembles more a woven textile.[15 16] The lamellae consist of flattened ribbon-like bundles of collagen fibrils and are about 0.2–2.5 μm thick and 0.5–250 μm wide.[15] The lamellae are aligned parallel to the surface and organised in layers, with a common orientation within the same layer. The diameter of the individual collagen fibres is about 25–35 nm and their interfibrillar distance about 20 nm.[18] The lamellar fibril bundles are partially interwoven within one layer but also between the layers, especially in the anterior stroma.[20] Similar to plywood,[12 18] the lamellae are arranged in layers with cross-angles between the lamellar layers. The lamellae of the anterior stroma are more obliquely arranged to each other compared with the more orthogonal cross-angles of the posterior stroma, with a preferential orientation along the superior–inferior and nasal–temporal meridians.[19] The collagen bands at the limbus are arranged in a more circular pattern fusing with the scleral ring.[19]

The interlamellar cohesion is maintained by interlacing lamellae providing structural tissue bridges, with molecular proteoglycan–glycosaminoglycan complexes acting like an interlamellar glue, keeping the stromal lamellae from falling apart, similar to mortar between bricks.[10 12 13 18] Interlamellar cohesion still allows a certain degree of physiological interlamellar sliding movement, especially under shear stress.[21] Interlacing, interlamellar fibril bundles were shown by Radner *et al* using microdissection and scanning electron microscopy.[15 16] The collagen fibril bundles often branch out in two or three subsidiary branches crossing through fissures between the branches of splitting fibre bundles creating an intensely interwoven meshwork.[10 15 16] The interlacing collagen lamellae should not be called crosslinks because this term should be reserved for chemical crosslinks. The details of the non-collagenous proteoglycan–glycosaminoglycan matrix are not yet fully clear. Proteoglycans are composed of a protein core with covalently attached sulphated glycosaminoglycan chains. In the cornea, the two predominant proteoglycans are keratan sulphate proteoglycans (lumican, keratocan and mimecan) and dermatan sulphate proteoglycans (decorin). The proteoglycans can be stained with quinolinic phthalocyanin or cuprolinic blue.[18] Müller *et al* showed that in the cornea hexagonally arranged collagen fibrils seem to be interconnected with their next-nearest neighbour by six proteoglycan proteins.[18] The cross-bridges between collagen fibrils that can be seen in quick freeze-deep etching specimens similar to steps of a ladder probably also represent proteoglycans.[18] Based on three-dimensional electron tomography, Lewis *et al* have proposed a more sophisticated and dynamic model in which the proteoglycans are attached to collagen fibrils but the interfibrillar bridges are randomly tilted and dynamic, producing an overall effect of a pseudo-hexagonal arrangement. In addition, the proteoglycan–glycosaminoglycan chains of the proteoglycans seem to join together in an anti-parallel non-covalent fashion forming partially overlapping, long complexes spanning the distance between more than two collagen fibrils.[22] The proteoglycans are considered to be crucial

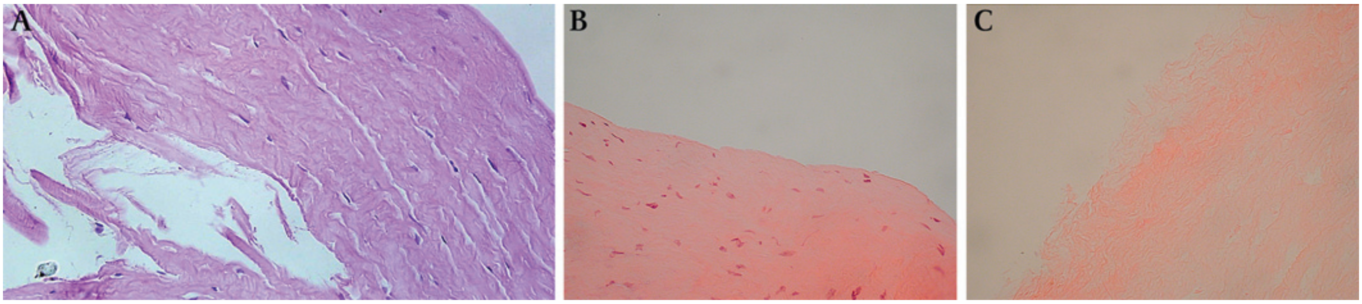

Figure 3 Histological section of the cleaving plane in (A) control (periodic acid–Schiff, ×400), (B) crosslinked, and (C) amylase-treated samples (haematoxylin and eosin, ×400). In all groups, torn lamellae could be seen. The tissue in the crosslinked group (B) looked more compact than the control corneas (A). The tissue in the α-amylase-treated sample (C) was much less tight, the collagen fibrils appeared wavy and the cell nuclei less basophilic.

for the resistance to compression and lateral stretch and for transparency.[11 18]

In this study, the interlamellar cohesive force was measured in the range of 0.139 N/mm centrally to 0.31 N/mm peripherally as reported previously by Smolek and McCarey, and by Maurice.[13 14] The tearing force peaks are probably related to abrupt tearing of interlacing collagen lamellae crossing the splitting plane.[13] The interlamellar connections appear to behave somewhat like a hook and loop fastener, and the interlamellar tearing effect resembles a zipper effect. Interestingly, after a microkeratome section the peaks cannot be found because of the mechanical severing of the interlamellar interlacing fibre bundles.[14] The absence of an effect of both chemical (formaldehyde) and physical (riboflavin/UVA) crosslinking on interlamellar cohesive force suggests that crosslinking in general mainly affects only the intra- and interfibrillar collagen molecule bonds. In crosslinking by riboflavin and UVA, recent studies by McCall *et al* have revealed that the collagen crosslinks induced by crosslinking are made up of covalent chemical bonds including carbonyl and amine groups,[5] whereas most formaldehyde crosslinks are methylene bridges between lysine and a peptide. The increase in collagen fibre diameter after crosslinking seems to be due to intrafibrillar collagen crosslinks[7] and the absence of oedema in the crosslinked anterior stroma in hydration studies due to interfibrillar collagen bonds.[8] It is possible that the interlamellar distance and the different orientations of the fibril lamellae prevent the creation of interlamellar collagen bonds. It is also possible that in untreated physiological corneas there are no significant interlamellar collagen bonds present, only interlacing lamellae and proteoglycans allowing a certain degree of interlamellar sliding movement.

On the other hand, it cannot be excluded that in vivo additional lamellar interlaces are induced by crosslinking during the postoperative corneal remodelling process, which could not be investigated in the present in vitro model and therefore is a methodical limitation of the present study. Concurrently, new needle-shaped hyper-reflective bands or bridges, which might be new interlamellar connections, have been observed using confocal microscopy in crosslinked human corneas.[23]

In contrast to Smolek and McCarey's studies on interlamellar cohesive force[13] and similar to the studies on the laser-assisted in situ keratomileusis (LASIK) flap adhesion,[14] we chose a tearing plane in the anterior stroma at about 200 μm depth because the crosslinking effect is mainly located in the anterior 350 μm of the stroma as has been demonstrated by studies on the hydration pattern,[8] keratocyte apoptosis,[2] collagen fibre diameter[7] and biomechanical stiffening effect.[6] The anterior localisation of the tearing plane might also explain the absence of the reduction in cohesive force in the oedematous samples because the specific architecture of the anterior stroma prevents major swelling.[8 10]

Amylase is known to digest proteoglycans selectively in connective tissue matrices.[24] Accordingly, the amylase-treated samples showed a significant reduction in cohesive force by about 31.5% due to the digestion of the proteoglycan bridges, whereas the collagen fibril interlaces were still intact, as demonstrated by the preserved spike pattern of the tearing curve (figure 2B) and the torn lamellae on histology (figure 3C). The loss of the proteoglycan bridges also explains the wavy appearance of the crimping collagen fibrils after amylase treatment.

It is an interesting question if it is possible to draw conclusions from this study regarding the pathogenesis of keratoconus. Interfibrillar and interlamellar slippage of corneal layers leading to the ultimate destruction of the lamellar configuration of the stroma[17] has been proposed as a pathogenetic hypothesis.[11 12] Similarly, in post-LASIK and post-photorefractive keratectomy (PRK) ectasia a reduction in lamellar number possibly due to interlamellar slippage and lamellar thinning due to interfibrillar slippage has been described ('interfibre fracture').[12] The present study has shown that there is probably no direct effect of crosslinking on interlamellar slippage. Given the apparent efficiency of crosslinking in the treatment of progressive keratoconus it might be cautiously concluded that the interfibrillar slippage effect is more important in the pathogenesis of keratoconus or post-LASIK ectasia or that the stabilisation of the interfibrillar collagen connections also reduces interlamellar slippage to a significant extent. In addition to collagen crosslink changes enzymatic proteoglycan degradations might play a role, as shown by the reduction in cohesive force in the amylase-treated group, and crosslinking of proteoglycans might increase their resistance to enzymatic degradation.

Our results explain the absence of major changes after corneal crosslinking treatment in applanation tonometry readings[1] and ocular response analyser[25] measurements, including corneal hysteresis or corneal resistance factor, because concentric anterior pressure is applied by these indentation methods causing interlamellar sliding movement[21] that is not influenced significantly by crosslinking, as shown by the present study.

In conclusion, our study has found that there is no significant effect of crosslinking on interlamellar cohesion, which is upheld mainly by interlacing collagen lamellae and the proteoglycans. Amylase-treated samples with digestion of proteoglycans had a reduction in interlamellar cohesive force by 31.5%, endorsing the significant role of the proteoglycans in interlamellar cohesion. Our results explain the absence of significant changes in tonometry and ocular response analyser measurements after corneal crosslinking and might help to further elucidate the pathogenesis of progressive keratoconus and post-LASIK ectasia.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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