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# **Interlamellar cohesion after corneal crosslinking using riboflavin and UVA**

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## **ABSTRACT**

**Aims:** Collagen crosslinking (CXL) treatment of progressive keratoconus using the photosensitizer riboflavin and UVA 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 mechanism. Therefore, the aim of this study was to assess the impact of CXL 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 hypoosmolar crosslinking group, the stromal swelling, the formaldehyde group, and the  $\alpha$ -amylase group. An anterior 9 x 4 mm strip of 400  $\mu$ m 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 hypoosmolar 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  $\alpha$ -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  $\alpha$ -amylase group the cohesive force was mainly decreased due to the digestion of proteoglycans. Crosslinking seems to stabilize only inter- and intrafibrillar but not interlamellar cohesion.

Corneal crosslinking treatment of progressive keratoconus using the photosensitizer riboflavin and ultraviolet A (UVA) was introduced by Wollensak<sup>1</sup> et al. from Germany in 2003 and has become increasingly popular in recent years.<sup>2,3</sup> Long-term results have confirmed the earlier positive results.<sup>3</sup>

The success of the new crosslinking method (CXL) in the treatment of progressive keratoconus (kc) is based primarily on its biomechanical stiffening effect stabilizing the corneal collagen fibril network and halting the progression of ectasia (“freezing”).<sup>2,4,5</sup> The crosslinking effect is strongest in the anterior stroma as found in numerous biomechanical,<sup>6</sup> histological<sup>2,7</sup> and hydration studies.<sup>8</sup>

Ultrastructurally, the collagen fiber diameter is increased by 12.2% in the anterior stroma after CXL concurrent with intrafibrillar collagen crosslinks<sup>7</sup>, and there is almost complete absence of hydration effects in the anterior crosslinked zone compatible with interfibrillar collagen crosslinks.<sup>8</sup> Recent studies have suggested that in keratoconus a reduction in the stromal cohesion may lead to interfibrillar or interlamellar slippage of collagen fibers.<sup>9-12</sup>

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

## **MATERIALS AND METHODS**

### **Sample preparation**

72 porcine eyes were retrieved from the local abattoir within 24 hours 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 x 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 (CXL) group, in which the porcine eyes were crosslinked according to the standard protocol of Wollensak<sup>1</sup> 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 physiologic saline solution) for 5 minutes before the irradiation, and every 5 minutes during the 30 minutes of irradiation using a commercial UVA diode system (Peschke Meditrade GmbH, Germany) with a surface irradiance of 3 mW/cm<sup>2</sup> and a focussing distance of 5 cm.
3. the hypoosmolar crosslinking group, which was treated like group 2 except for applying a hypoosmolar 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 physiologic saline for 24 hours 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 hours.
6. the  $\alpha$ -amylase group, in which the corneas were kept in an aqueous solution of  $\alpha$ -amylase (from *Aspergillus oryzae*,  $\geq 800$  FAU/g, Sigma-Aldrich) for 72 hours at room temperature to digest the proteoglycans.

## **Pachymetry**

After treatment, the thickness of the initially 400  $\mu$ m thin flaps was determined using ultrasound pachymetry (Pachette, Technomed).

## **Interlamellar cohesive force measurements**

4 x 9 mm anterior corneal 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; fig 1A and 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 newtons (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 (fig 2 A and B, table1). For statistical analysis, the p value of the biomechanical data was calculated using a Student's t-test for the comparison with the control group .

## **Histology**

Characteristic samples from each treatment group were fixed in 4% formaldehyde for three days and embedded in paraffin. 4  $\mu$ m thin paraffin cross-sections were cut and stained with hematoxylin-eosin (H & E) and periodic acid-Schiff (PAS).

## **RESULTS**

### **Gross appearance and pachymetry**

The flaps in all treatment groups except for the group 3 and 4 with edema appeared transparent. The swelling effect in group 3 and 4 was rather weak because only the anterior 400  $\mu$ m of the stroma were used, which is rather resistant to swelling. After swelling, the mean thickness of the hypoosmolar group 3 was increased to  $442 \pm 23$   $\mu$ m and of the physiologic saline group 4 to  $433 \pm 27$   $\mu$ m. The pachymetry readings for the control group were  $402 \pm 18$   $\mu$ m, the CXL group  $398 \pm 12$   $\mu$ m, the formaldehyde group  $395 \pm 7$   $\mu$ m and the  $\alpha$ - amylase group  $376 \pm 22$   $\mu$ m. The samples from group 2 and 3 had a yellowish tint due to the applied riboflavin solution (fig. 1A). The  $\alpha$ - amylase treated samples appeared more transparent than the flaps of all the other groups including the controls (fig. 1 B).

### **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 localized fluctuations in required tearing force (fig 2 A and B) were registered.<sup>13,14</sup> Only the values of the  $\alpha$ -amylase group were statistically significantly different from the control group, with a decrease in cohesive force by -31.5% ( $p < 0.0001$ ; table 1). The slight increase in interlamellar cohesive force after CXL by 5.7% was not statistically significant ( $p < 0.31$ ). The number of samples required to exclude a type II error for the CXL changes would be 60 samples for both the CXL 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 (fig. 3A) and even more so in the CXL group, the tissue appeared more compacted (fig 3 B) and the lamellae straightened, in the edematous group 3 and 4 only a moderate degree of stromal edema was seen, in the  $\alpha$ -amylase group the collagen lamellae appeared rather wavy, there was some edema, and the cell nuclei appeared less basophilic (fig 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 remarkable and surprising. Apparently, collagen crosslinks are mainly intrafibrillar and interfibrillar but not interlamellar. On the other hand, the digestion of proteoglycans by  $\alpha$ -amylase led to a significant decrease of interlamellar

cohesion by -31.5% demonstrating the important role of the proteoglycan-glycosaminoglycan complexes for interlamellar stabilization.

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 just like a stack of 300 sheets of copy paper that can slide to each other, when subjected to shear. Detailed studies using scanning electron microscopy<sup>15,16,17</sup>, microdissection analysis<sup>15,16</sup>, transmission electron microscopy<sup>18</sup>, polarised light microscopy or X-ray diffraction<sup>19</sup> could demonstrate that the cornea resembles more a woven textile.<sup>15,16</sup> The lamellae consist of flattened ribbon-like bundles of collagen fibrils and have about 0.2-2.5  $\mu\text{m}$  thickness and 0.5 - 250  $\mu\text{m}$  width.<sup>15</sup> The lamellae are aligned parallel to the surface and organized in layers, with a common orientation within the same layer. The diameter of the individual collagen fibers is about 25-35 nm and their interfibrillar distance about 20nm.<sup>18</sup> The lamellar fibril bundles are partially interwoven within one layer but also between the layers especially in the anterior stroma.<sup>20</sup> Similar to plywood,<sup>12,18</sup> 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 to the more orthogonal crossangles of the posterior stroma with a preferential orientation along the superior-inferior and nasal-temporal meridians.<sup>19</sup> The collagen bands at the limbus are arranged in a more circular pattern fusing with the scleral ring.<sup>19</sup>

The *interlamellar cohesion* is maintained by interlacing lamellae providing structural tissue bridges, and molecular proteoglycan-glycosaminoglycan (GAG's) complexes acting like an interlamellar glue, keeping the stromal lamellae from falling apart similar to mortar between bricks.<sup>10,12,13,18</sup> Interlamellar cohesion still allows a certain degree of physiological interlamellar sliding movement, especially under shear stress.<sup>21</sup> Interlacing, interlamellar fibril bundles could be nicely shown by Radner et al. using microdissection and scanning electron microscopy.<sup>15,16</sup> The collagen fibril bundles often branch out in two or three subsidiary branches crossing through fissures between the branches of splitting fiber bundles creating an intensely interwoven meshwork.<sup>10,15,16</sup> 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 (GAG) matrix are not yet fully clear. Proteoglycans (PG's) are composed of a protein core with covalently attached sulfated glycosaminoglycan (GAG) chains. In the cornea, the two predominant proteoglycans are keratan sulfate proteoglycans (lumican, keratocan, and mimecan) and dermatan sulfate proteoglycans (decorin). The proteoglycans can be stained with quinolinic phthalocyanin (QP) or cuproinic blue (CB).<sup>18</sup> Müller et al. from Holland could show that in cornea hexagonal arranged collagen fibrils seem to be interconnected with their next-nearest neighbour by six proteoglycan proteins.<sup>18</sup> The cross-bridges between collagen fibrils that can be seen in quick freeze-deep etching specimens similar like steps of a ladder probably also represent proteoglycans.<sup>18</sup> Based on 3D-electron tomography, Lewis et al. from Cardiff have proposed a more sophisticated and dynamic model in which the PG's are attached to collagen fibrils but the interfibrillar bridges are randomly tilted and dynamic producing an overall effect of a pseudo-hexagonal arrangement. Also, the GAG chains of the PG's seem to join together in an antiparallel noncovalent fashion forming partially overlapping, long complexes spanning the distance between more than two collagen fibrils.<sup>22</sup> The PG's are considered to be crucial for the resistance to compression and lateral stretch and for transparency.<sup>11,18</sup>

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 et al. and Maurice.<sup>13,14</sup> The tearing force peaks are probably related to abrupt tearing of interlacing collagen lamellae crossing the splitting plane.<sup>13</sup> 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 fiber bundles.<sup>14</sup> 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 CXL are made up of covalent chemical bonds including carbonyl and amine groups<sup>5</sup> whereas most formaldehyde crosslinks are methylene bridges between lysine and a peptide. The increase in collagen fiber diameter after crosslinking seems to be due to intrafibrillar collagen crosslinks<sup>7</sup> and the absence of edema in the crosslinked anterior stroma in hydration studies due to interfibrillar collagen bonds.<sup>8</sup> Possibly, the interlamellar distance and the different orientations of the fibril lamellae prevent the creation of interlamellar collagen bonds. Maybe, also in untreated physiological corneas no significant interlamellar collagen bonds are 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 remodeling 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 hyperreflective bands or bridges have been observed using confocal microscopy in crosslinked human corneas which might be new interlamellar connections.<sup>23</sup>

In contrast to Smolek's studies on interlamellar cohesive force<sup>13</sup> and similar to the studies on the LASIK flap adhesion<sup>14</sup> we chose a tearing plane in the anterior stroma at about 200  $\mu\text{m}$  depth because the crosslinking effect is mainly located in the anterior 350  $\mu\text{m}$  of the stroma as has been nicely demonstrated by studies on the hydration pattern,<sup>8</sup> keratocyte apoptosis,<sup>2</sup> collagen fiber diameter<sup>7</sup>, and biomechanical stiffening effect.<sup>6</sup> The anterior localization of the tearing plane might also explain the absence of the reduction in cohesive force in the edematous samples because the specific architecture of the anterior stroma prevents major swelling.<sup>8,10</sup>

Amylase is known to digest selectively proteoglycans in connective tissue matrices.<sup>24</sup> 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 (fig 2B) and the torn lamellae on histology (fig 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<sup>17</sup> has been proposed as a pathogenetic hypothesis.<sup>11-12</sup> Similarly, in post-LASIK and post-PRK ectasia a reduction in lamellar number possibly due to interlamellar slippage and lamellar thinning due to interfibrillar slippage has been described („interfiber fracture“).<sup>12</sup> 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 stabilization 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 PG's might increase their resistance to enzymatic degradation.

Our results can nicely explain the absence of major changes after corneal crosslinking treatment in applanation tonometry readings<sup>1</sup> and ocular response analyzer (ORA)<sup>25</sup> measurements including corneal hysteresis (CH) or corneal resistance factor (CRF), because concentric anterior pressure is applied by these indentation methods causing interlamellar sliding movement<sup>21</sup>, which 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% corroborating the significant role of the proteoglycans in interlamellar cohesion. Our results explain the absence of significant changes in tonometry

and ORA measurements after corneal crosslinking and might help to further elucidate the pathogenesis of progressive keratoconus and post-LASIK ectasia.

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## **FIGURES**

**Figure 1** Cohesive force measurements of (A) riboflavin/UVA crosslinked and (B)  $\alpha$ -amylase treated anterior porcine corneal flaps being torn apart at 200  $\mu\text{m}$  depth by the clamps of a biomaterial testing machine. Note the yellowish tint of the crosslinked samples due to the photosensitizer riboflavin and the increased transparency of the  $\alpha$ - amylase treated sample.

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

**Figure 3** Histologic section of the cleaving plane in A control (PAS stain, x 400), B crosslinked, and C amylase-treated samples (H&E, x400). 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  $\alpha$ -amylase-treated sample (C) was much less tight, the collagen fibrils appeared wavy and the cell nuclei less basophilic.























