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Thermomechanical Behavior of Collagen-Cross-Linked Porcine Cornea

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Cross-linking · Cornea · Thermomechanical behavior · Riboflavin · Ultraviolet A

Abstract
Purpose: Collagen cross-linking using combined riboflavin/UVA treatment has been shown to increase the biomechanical rigidity of the cornea and has been used successfully for the treatment of progressive keratoconus. From morphological and biochemical investigations, a different degree of cross-linking for the anterior and posterior stroma by the treatment is suggested. The present study was undertaken to better evaluate this effect by testing the thermomechanical behavior. ***Methods:*** Ten 10 × 5 mm corneal strips from porcine cadaver eyes enucleated within 5 h post mortem were cross-linked using the photosensitizer riboflavin and UVA irradiation (370 nm, irradiance = 3 mW/cm^2) for 30 min and compared to ten untreated corneal strips and ten corneal strips cross-linked with 0.1% glutaraldehyde. The temperature in a water bath was raised from 60 to 95°C with temperature increments of 1°C per minute. The hydrothermal shrinkage of the corneal strips was measured in 2.5°C steps using a micrometer. In addition, six 10-mm whole corneal buttons were cross-linked with riboflavin/UVA and immersed into water at 70 or 75°C. ***Results:*** The maximal hydrothermal shrinkage for the untreated control specimens and the posterior portion of the riboflavin/UVA-treated corneas was at 70°C, for the anterior portion of the cornea cross-linked by riboflavin/UVA at 75°C and for glutaraldehyde-cross-linked cornea at 90°C. In the cross-linked corneal buttons, a typical mushroom-like shape was observed at 70°C and a cylinder shape at 75°C. ***Conclusions:*** The different degree of collagen cross-linking in the corneal stroma after riboflavin/UVA treatment is reflected by the differences in the maximal shrinkage temperature of the anterior and posterior portion. Therefore, in the corneas cross-linked with riboflavin/UVA a higher shrinkage temperature was observed for the anterior portion of the cornea (75°C) compared to the posterior stroma (70°C) due to the higher degree of cross-linking of the anterior stroma. The anterior localization of the cross-linking effect is advantageous for the endothelium and for the preservation of the anterior corneal curvature.

Introduction

Collagen cross-linking in the cornea using UVA and the photosensitizer riboflavin has recently been developed for stiffening the cornea. In a prospective clinical

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pilot study including 22 patients with moderate or advanced progressive keratoconus, and with a follow-up time of up to 4 years, the progression of keratoconus was stopped in all treated eyes. Regression with a reduction of the maximal keratometry readings by 2 diopters was achieved in 70% of patients [1]. Therefore, collagen cross-linking might become a new way to stop progressive keratoconus. Other possible clinical applications of corneal collagen cross-linking lie in the field of refractive surgery, corneal ulcers, stromal melting and thinning.

In biomechanical stress-strain measurements, we could show a significant increase in corneal rigidity by about 300% in human corneas and 75% for porcine cornea after the cross-linking treatment [2]. In addition, increased resistance to enzymatic digestion by collagenases [3] was demonstrated. However, from studies on the changes in keratocytes [4] and collagen fiber diameter [5] we know that the cross-linking effect is maximal only in the anterior 300 μm of the cornea due to the massively increased absorption of UVA light by riboflavin.

The aim of the present study was to demonstrate the difference in the degree of cross-linking between the anterior and posterior stroma by investigating the thermomechanical shrinkage behavior because cross-linking results in less shrinkage after heat denaturation.

Material and Methods

Porcine Corneal Strips

Thirty fresh porcine cadaver eyes with intact epithelium and clear corneas were retrieved from the local slaughterhouse within 3–5 h post mortem. The eyes were de-epithelialized mechanically by a cotton swab. The cornea-scleral ring was removed using a pair of scissors. One corneal strip of 5 mm width and 10 mm length was cut from the center of each cornea in a superior-inferior orientation, using a double-bladed scalpel especially designed for this purpose.

Ten specimens were used for each of the following treatment groups: (1) corneal strips treated with riboflavin/UVA irradiation were bluntly separated into an anterior 400-μm (1a) and a posterior 400-μm (1b) portion; (2) corneal strips treated with 0.1% glutaraldehyde for 30 min, and (3) untreated control corneas.

Whole Corneal Buttons

In addition, three 10-mm whole corneal porcine buttons cross-linked with riboflavin/UVA were immersed in water at 70°C and another three at 75°C.

Cross-Linking Treatment

Starting 5 min before the treatment, 0.1% riboflavin (= vitamin B_2) photosensitizer solution (10 mg riboflavin 5′-phosphate in 10 ml 20% dextran T-500) was dropped onto the corneal strips at 5-min intervals. UVA irradiation (370 nm) was applied using a double UVA diode (Roithner Lasertechnik, Vienna, Austria) with an irradiance of 3 mW/cm^2 at a distance of 1 cm from the cornea for 30 min. In addition, chemical cross-linking by 0.1% glutaraldehyde for 30 min was performed in the second treatment group.

Maximal Shrinkage Measurement

A water bath with an attached thermostat was heated gradually from 60 to 95°C at a linear rate of approximately 1°C per minute. The hydrothermal linear shrinkage was measured in 2.5°C steps. For the determination of the shrinkage, the corneal strips were taken out of the water bath for about 20 s, oriented longitudinally and their length measured using a micrometer (Feinmesszeugfabrik Suhl, Germany) with an accuracy of 10 μm.

Histology

For histological examination, 2 × 2 corneal buttons heated at 70 and 75°C were fixed in 4% formalin and embedded in paraffin. Four-micrometer-thick sections were stained for hematoxylin/eosin and examined in a Zeiss light microscope (Axiomat) including a polarization filter.

Statistical Evaluation

The maximal shrinkage temperature data found for treated and untreated corneas were statistically compared using one-way ANOVA (SPSS statistical program from SPSS, Munich, Germany).

Results

Maximal Shrinkage Measurements (fig. 1)

The shrinkage behavior for the various subgroups was as follows:

(1a) Anterior portion of corneal strips treated with riboflavin/UVA irradiation: beginning of shrinkage at 67.5°C, maximal shrinkage at 75.0 ± 1.2°C.

(1b) Posterior portion of corneal strips treated with riboflavin/UVA irradiation: beginning of shrinkage at 63°C, maximal shrinkage at 71.2 ± 0.8°C.

(2) Corneal strips treated with 0.1% glutaraldehyde: beginning of shrinkage at 77.5°C, maximal shrinkage at 89.7 ± 1.7°C.

(3) Untreated control corneas: beginning of shrinkage at 62.5°C, maximal shrinkage at 70.3 ± 0.9°C.

The differences in maximal shrinkage temperature were statistically significant between all groups with a level of significance $p = 0.001$. There was no statistically significant difference between the untreated control strips and the posterior portion of the cross-linked corneas.

Whole Corneal Buttons

After thermal exposure, the cross-linked corneal buttons displayed a mushroom configuration at 70°C with only the posterior portion shrunken and a cylinder shape at 75°C with both anterior and posterior portions contracted due to heat degeneration (fig. 2). The stroma be-

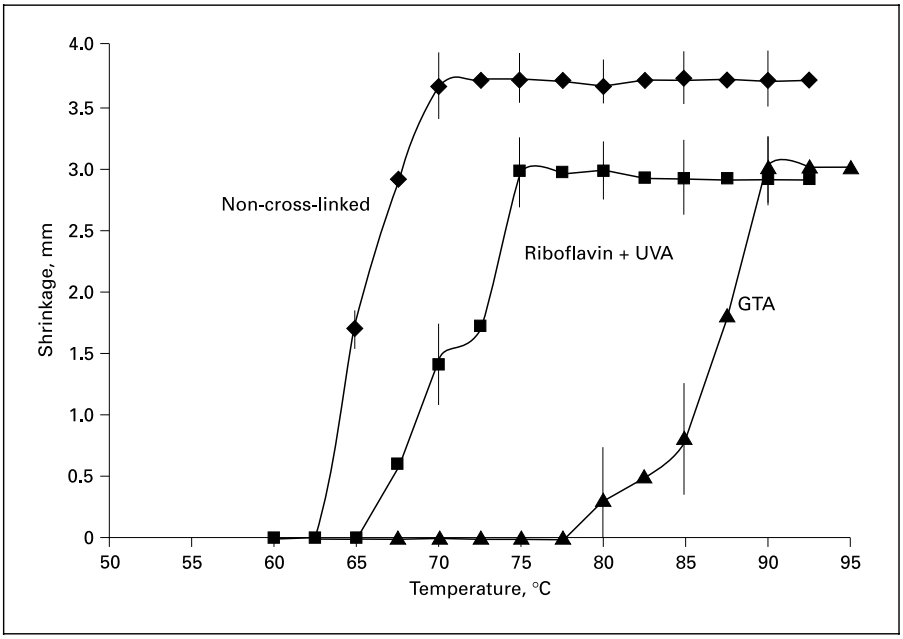

Fig. 1. Corneal shrinkage as a function of temperature with maximum shrinkage for untreated controls at 70 °C, specimens cross-linked with riboflavin and UVA at 75 °C and specimens cross-linked with 0.1% glutaraldehyde at 90 °C.

came opaque at the beginning of shrinkage. Maximum shrinkage led to a corresponding increase in stromal thickness of the affected layer.

Histology

In the mushroom-shaped specimens, a clear-cut difference was found between the anterior, nonshrunken cross-linked portion with preserved birefringence of the collagen fibers and the posterior, contracted portion with homogenized denatured collagen fibers and loss of birefringence (fig. 3a, b). In the cylinder-shaped specimen, all layers displayed degenerated collagen fibers with loss of birefringence.

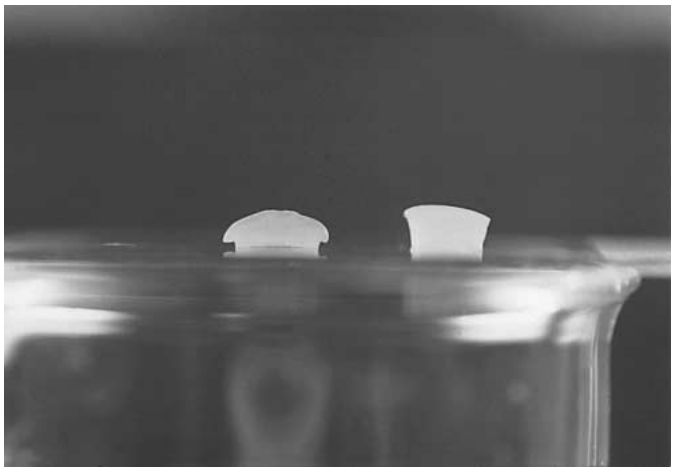

Fig. 2. After thermal exposure, the cross-linked corneal buttons displayed a mushroom configuration at 70 °C (left) with only the posterior portion shrunken and a cylinder shape at 75 °C (right) with both anterior and posterior portions contracted due to heat degeneration.

Discussion

The degree of collagen cross-linking was reflected by the height of the maximum shrinkage temperature, being 70 °C for the untreated cornea, 75 °C for the cornea cross-linked with riboflavin/UVA and 90 °C for cornea cross-linked with glutaraldehyde.

The corneal buttons nicely demonstrated the difference in the degree of cross-linking between the anterior and posterior portion leading to a mushroom shape at 70 °C when only the posterior non-cross-linked portion is contracted (fig. 2a) and to a cylinder shape at 75 °C (fig. 2b) when both anterior and posterior portions are denatured by heat. Cum grano salis one could compare the phenomenon to the different heat expansion in bimetals. Other parameters indicating a different degree of cross-linking between the anterior and posterior portions of the cornea after riboflavin/UVA treatment are an increase of collagen fiber diameter mainly in the anterior portion [5], preferred preservation of the anterior portion

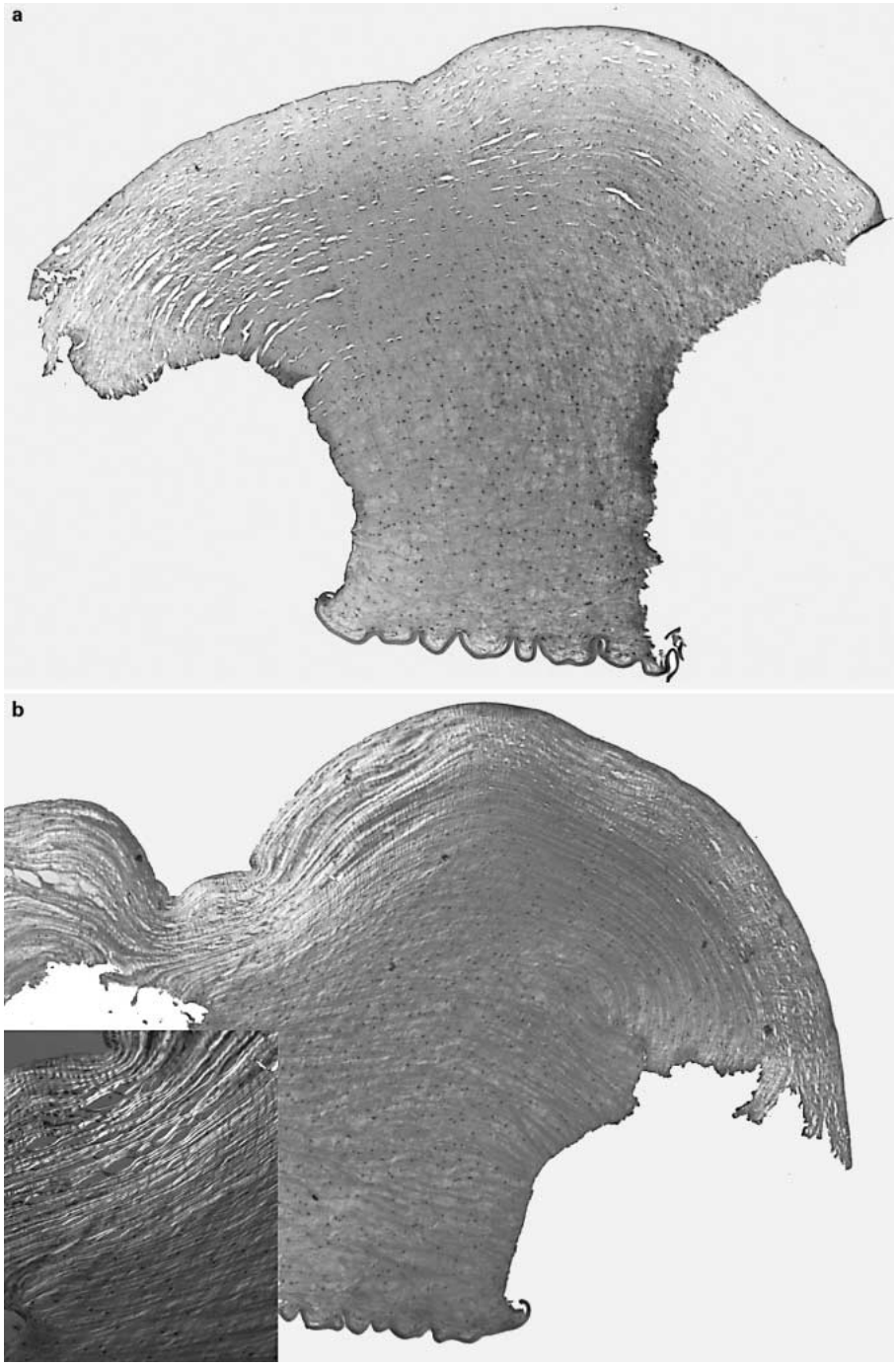

Fig. 3. Photomicrograph of a mushroom-shaped specimen at 70 °C with anterior well-preserved collagen fibers and shrunken homogenized fibers in the posterior portion (**a**, × 40) and loss of birefringence in the posterior contracted portion while it is preserved in the anterior cross-linked portion (**b**, × 40). **Inset** Higher magnification (× 200).

after enzymatic digestion [3] and the anterior localization of the treatment-related keratocyte damage [4].

The difference in the degree of cross-linking is caused by the rapid loss of UVA irradiance across the cornea due to riboflavin-enhanced UVA absorption. The anterior localization of the cross-linking effect is advantageous for clinical use because the endothelium is effectively spared from UVA-related damage and the anterior portion of the cornea is more important for the maintenance of the overall corneal curvature and for the optical refractive effect [6].

Collagen shrinkage is a consequence of heat-induced collagen denaturation. More cross-linking results in less shrinkage as has been shown for glutaraldehyde-treated bovine pericardium [7] and collagen membranes cross-linked with glutaraldehyde or DMS [8]. It depends not only on the nature but also on the density of the collagen cross-links [9]. The shrinkage effect is used therapeutically in thermokeratoplasty [10] and also for treating capsular laxity in joints [11, 12]. Our values found for the maximum shrinkage correlate well with other studies. So the shrinkage temperature for collagen in patellar tendons was 70°C [13] and 84.5°C for glutaraldehyde-treated bovine pericardium [7].

Thermal shrinkage of collagen is caused by the denaturation and uncoiling of the triple helix [9], and also the loss of birefringence is due to the unwinding of the collagen triple helix [12]. When heated above a critical temperature level collagen fibers undergo a phase transition from a highly organized semicrystalline state to a melted gel of randomly oriented polypeptide chains [12], sometimes called hyalinization, in which the covalent bonds between the collagen molecules are supposed to be broken. Shrinkage is also dependent on the collagen type and its content of hydroxyproline and proline being relatively low for corneal type I collagen [11].

The present study has clearly demonstrated the cross-linking effect in the anterior stroma as demonstrated by the increased maximum shrinkage temperature of the cross-linked collagen in the anterior portion of the cross-linked cornea. The limitation of the cross-linking effect to the anterior stroma is advantageous for the treatment because the endothelium is spared and the stiffening effect is greater at the surface and in the anterior portion of the cornea, which is most important for stabilizing the corneal curvature.

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