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Abstract

Purpose. Collagen-crosslinking using combined riboflavin/UVA treatment has been developed by us as a new treatment for keratoconus by stiffening the collagenous matrix. Recently, we have started to use the same method for the treatment of corneal ulcers. The aim of the present study was to evaluate the influence of the crosslinking treatment on the resistance of the cornea against enzymatic degradation.

Methods. 60 enucleated porcine eyes were treated with the photosensitizer riboflavin and UVA-irradiation (370 nm; irradiance of 1, 2 or 3 mW/cm^2) for 30 minutes and compared with 20 untreated control eyes. After crosslinking treatment, the corneal buttons were trephined and exposed to pepsin, trypsin and collagenase solutions. The extent of the corneal digestion was monitored daily. Selected cases were examined by light microscopy.

Results. The corneal buttons crosslinked with riboflavin/UVA at 3 mW/cm^2 were dissolved only by day 13 following pepsin digestion and by day 14 following collagenase treatment versus 6 days in the untreated control corneas. Digestion by trypsin was observed on day 5 in buttons crosslinked at 3 mW/cm^2 compared to day 2 in the control corneas. Microscopically, a prolonged preservation especially of the anterior portion of the crosslinked corneas could be demonstrated.

Conclusions. Photochemical crosslinking of the cornea using riboflavin and UVA results in a markedly increased resistance versus collagen digesting enzymes. The findings support the use of the new method in the treatment of corneal ulcers.

Introduction

Collagen-crosslinking of the cornea using UVA and the photosensitizer riboflavin has been developed by us recently for stiffening the collagenous matrix of the cornea. Using this method photodynamic crosslinking is achieved when the photosensitizer riboflavin excited by UVA at its absorption peak of 370 nm creates free radicals leading to physical crosslinking of collagen fibers[1] a process which can also be observed in the formation of cataract where endogenous photosensitizers including riboflavin induce crosslinking of the crystallin proteins leading to increased stiffness of the lens and to the formation of high molecular weight crystallins.[2]

In a clinical pilot study including eyes with progressive keratoconus and with a follow-up time of up to 4 years the progression of keratoconus could be stopped in all patients treated with riboflavin/UVA and regression with a reduction of the maximal keratometry readings by 2 diopters was achieved in 70%.[3] Therefore, collagen crosslinking might be a new way for stopping the progression of keratectasia in keratoconus patients.

Recently, we have started using increasingly this new crosslinking method for the treatment of corneal melting processes and ulcers which must have, however, a minimal residual stromal thickness of 400 μm because of the risk involved for the endothelium.[4,5] Especially in peripheral

corneal melting processes due to rheumatoid arthritis we have achieved good results using crosslinking by riboflavin/UVA. Collagenases and other metalloproteinases are known to play an important role in the pathogenesis of corneal ulceration[6,7] and to some extent also in keratoconus.[8] Therefore, collagenase inhibitors like Ca-EDTA are often used in the treatment of corneal ulceration.[6,9] Especially in corneal ulceration, the biochemical effect by collagen crosslinking might be more important than the biomechanical effect (Fig. 1).[10]

Therefore, this study was undertaken to assess the influence of the crosslinking treatment on the resistance of corneas crosslinked by riboflavin/UVA against digestion by collagenase solution. In addition, pepsin and trypsin digestion was tested because the three enzymes are usually part of the enzyme panel to assess the efficacy of a crosslinking procedure.[11]

Materials and methods

Eye specimens

80 enucleated porcine eyes with clear corneas were obtained from the local abattoir within 8 hours of death. For each of the three enzymatic treatment groups with either pepsin, collagenase or trypsin digestion 20 eyes were divided into the following subgroups:

1. non-crosslinked control group (5 eyes)
2. riboflavin plus irradiance level: $1 \, mW/cm^2$ (5 eyes)
3. riboflavin plus irradiance level: $2 \, mW/cm^2$ (5 eyes)
4. riboflavin plus irradiance level: $3 \, mW/cm^2$ (5 eyes)

Crosslinking of collagen

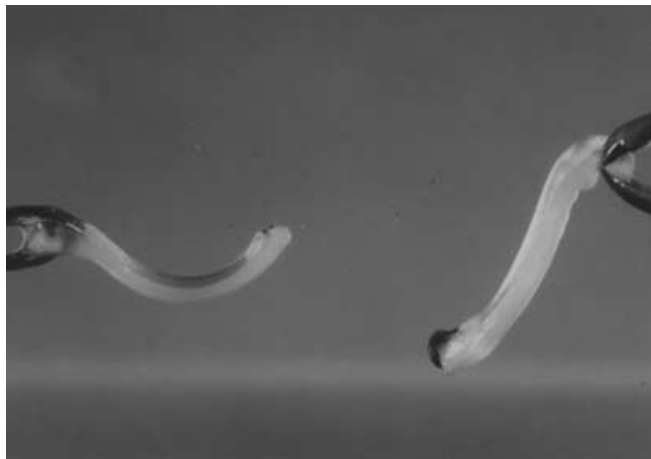The corneal epithelium of the otherwise intact globe was completely scraped off mechanically using a blunt hockey knife. After the debridement, riboflavin photosensitizer solution containing 0.1% riboflavin-5-phosphate (Fig. 2) and 20% dextran T 500 was dropped onto the cornea starting 5 minutes before the irradiation and every 5 minutes during the irradiation. The specimens were irradiated with UVA-light (370 nm) using two UVA-diodes (Roithner Lasertechnik, Viena, Austria) with an irradiance of 1, 2 or $3 \, mW/cm^2$ for 30 minutes in a distance of 1 cm from the cornea. The control eyes were deepithelialized, irrigated with the dextran/riboflavin-solution for 30 minutes but not irradiated. After irradiation, 10 mm corneal buttons were trephined and placed into plastic containers with the different enzyme solutions.

Enzymatic digestion

Pepsin assay

The pepsin solution was made up of 1 g purified pepsin (EC 3.4.23.1, Sigma, Munich, 2500 U/mg) in 10 ml 0.1 M HCL at pH 1.5 being the pH-optimum for pepsin activity.

Trypsin assay

The corneal buttons were placed into plastic containers with a solution made up of 1 g trypsin (EC 3.4.21.4, Sigma, Munich, Germany) in 10 ml PBS-buffer at pH 7.5 being the pH optimum for trypsin activity. As trypsin digestion did not occur in non-denatured corneas (n = 20) additional crosslinked and control corneas (n = 20) were exposed to trypsin after prior heat denaturation in boiling water at 100°C for 10 min.
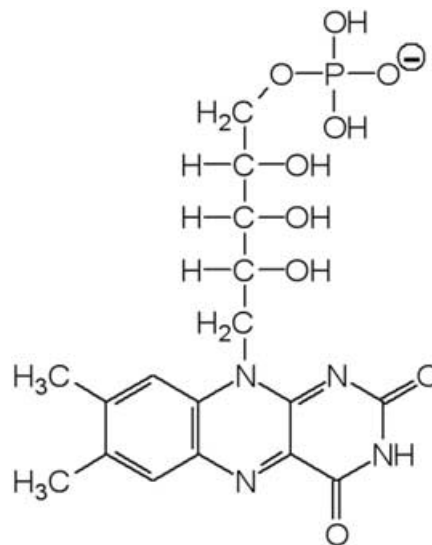

Figure 1. Increased stiffness of porcine cornea treated with riboflavin/UVA (left) compared to untreated cornea (right).

Figure 2. Structural formula of riboflavin (MW 456).

Collagenase assay

The crosslinked and the control buttons were placed into a 0.1% bacterial collagenase A solution (100 U/g or 0.1 U/ml) in PBS at pH 7.5 (EC 3.4.24.3 from Clostridium histolyticum, Roche, Mannheim, Germany).

Changes of button diameter

After crosslinking treatment and exposure to the enzyme solutions the buttons were monitored for their diameter daily and were photographed as well (Figs. 3, 4). The thickness of the button could not be used as a reliable parameter because in the beginning the untreated corneas became swollen in the vertical direction leading to increased thickness compared to the crosslinked buttons whereas there was no increase in diameter so that the diameter was the same for all specimens in the beginning of the digestion.

Statistical evaluation

The statistical analysis of the resorption times of the button (Table 1) diameter from 100–50% reduction and from 50–10% reduction was performed using ANOVA and an additional post-hoc analysis (Bonferroni's test). All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS GmbH, Munich, Germany).

Histology

For histological evaluation, selected buttons from the various treatment groups were fixed in 4% formalin on various days and embedded in paraffin. 4 µm thick paraffin sections were stained with hematoxylin/eosin and PAS (Fig. 5A + B) and assessed using a light microsope (Axioskop, Zeiss, Germany).

Results

Enzymatic digestion

Pepsin treatment (Figs. 3A, 4A)

Complete digestion was noted in non-crosslinked controls after 6 days, in riboflavin plus UVA-irradiance of $1 \, mW/cm^2$ after 6 days as well, in riboflavin plus UVA-irradiance of $2 \, mW/cm^2$ after 11 days, in riboflavin plus UVA-irradiance of $3 \, mW/cm^2$ after 13 days (Fig. 3A).The anterior curvature of the cornea with UVA-irradiance of 2 and $3 \, mW/cm^2$ was maintained in the first 7 days (Fig. 6). The transparency was lost in all specimens during the first day and was associated with significant swelling up to 4 mm in the first days.

Trypsin treatment (Fig. 3B)

The non-heat-denatured corneal buttons (n = 20) could not be digested by trypsin. After heat denaturation, complete
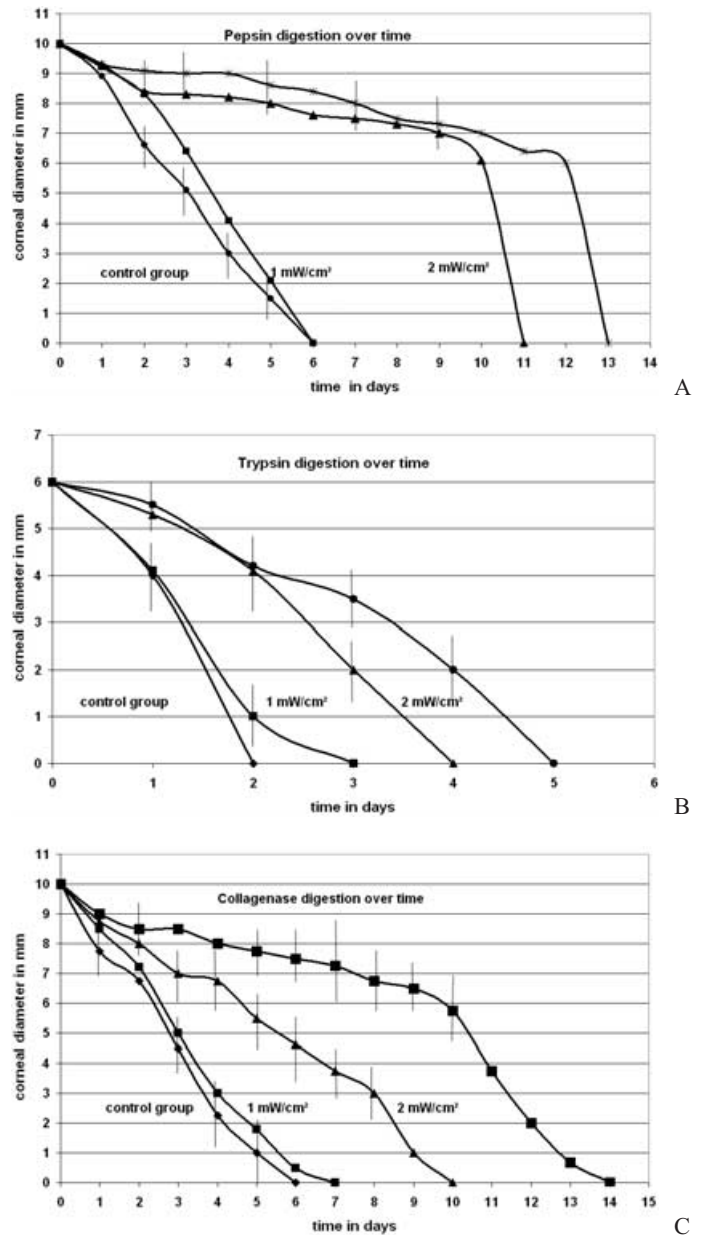

A

B

C

Figure 3. (A) Change of corneal diameter over time after pepsin digestion. (B) After heat denaturation at 100°C and trypsin digestion. (C) After collagenase digestion.

digestion was noted in non-crosslinked controls after 2 days, in riboflavin plus UVA-irradiance of $1 \, mW/cm^2$ after 3 days, in riboflavin plus UVA-irradiance of $2 \, mW/cm^2$ after 4 days, in riboflavin plus UVA-irradiance of $3 \, mW/cm^2$ after 5 days. (Fig. 3B).

Collagenase treatment (Figs. 3C, 4B)

Complete digestion was found in non-crosslinked controls after 6 days, in riboflavin plus UVA-irradiance of $1 \, mW/cm^2$ after 7 days, in riboflavin plus UVA-of irradiance $2 \, mW/cm^2$ after 10 days, in riboflavin plus UVA-of irradiance

Table 1. Time needed for the reduction in button diameter, in the range from 100–50% tissue reduction and from 50 to 10% tissue reduction (in days).

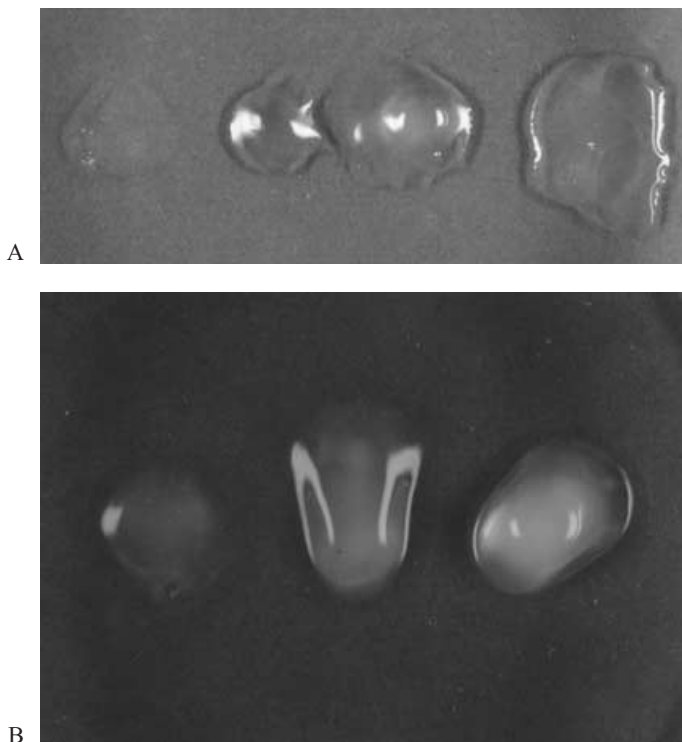|	Duration of reduction from 100 to 50%		Duration of reduction from 50 to 10%	
	mean ± S.D.	*P*-value	mean ± S.D.	*P*-value
collagenase				
control	2.81 ± 0.8		2.4 ± 0.5	
1 mW/cm^2	3.2 ± 0.8	1.0	2.6 ± 0.9	1.0
2 mW/cm^2	5.6 ± 0.5	0.001	3.4 ± 0.9	0.26
3 mW/cm^2	10.5 ± 0.5	0.001	2.2 ± 0.4	1.0
trypsin				
control	1.2 ± 0.4		0.6 ± 0.5	
1 mW/cm^2	1.4 ± 0.5	1.0	1.2 ± 0.4	0.84
2 mW/cm^2	2.6 ± 0.9	0.03	1.2 ± 0.4	0.82
3 mW/cm^2	3.2 ± 0.8	0.02	1.6 ± 0.9	0.12
pepsin				
control	3.0 ± 0.7		2.4 ± 0.5	
1 mW/cm^2	3.6 ± 0.5	1.0	1.8 ± 0.8	0.919
2 mW/cm^2	10.4 ± 0.5	0.001	0.6 ± 0.5	0.002
3 mW/cm^2	12.4 ± 0.9	0.001	0.4 ± 0.5	0.001

Figure 4. (A) Untreated control and crosslinked (1 mW/cm^2, 2 mW/cm^2, 3 mW/cm^2) buttons (from left to right) after 3 days of *pepsin* digestion. (B) Untreated control and crosslinked (2 mW/cm^2, 3 mW/cm^2) buttons (from left to right) after 3 days of *collagenase* digestion.

3 mW/cm^2 after 14 days (Fig. 3C). The difference in collagenase digestion between the various UVA-irradiances was most prominent and statistically significant in the 100–50% resorption range (Table 1) whereas in the 50–10% resorption range there was no statistically significant effect. The anterior curvature of the cornea with UVA irradiance of 3 mW/cm^2 was maintained in the first 5 days.

Histology

In the non-crosslinked control buttons digestion was present on all margins with no preference for any location. Descemet's membrane was dissolved on day 2 in the crosslinked buttons exposed to pepsin, trypsin and to collagenase. The digestion of the crosslinked buttons started in the posterior half of the stroma including Descemet's membrane while the stroma remained intact in its anterior portion in the first days of digestion (Fig. 5B).

Discussion

In this study, we have found an impressive doubling in the digestion time following pepsin, trypsin and collagenase digestion in corneas crosslinked with riboflavin and UVA at 3 mW/cm^2 compared to the controls. This finding reflects the biochemical effect of the crosslinking treatment in addition to the already known biomechanical effect.[10]

Resistance to collagenase digestion is an essential aspect in the efficacy of the crosslinking treatment in corneal ulceration because increased collagenolytic activity is one of the
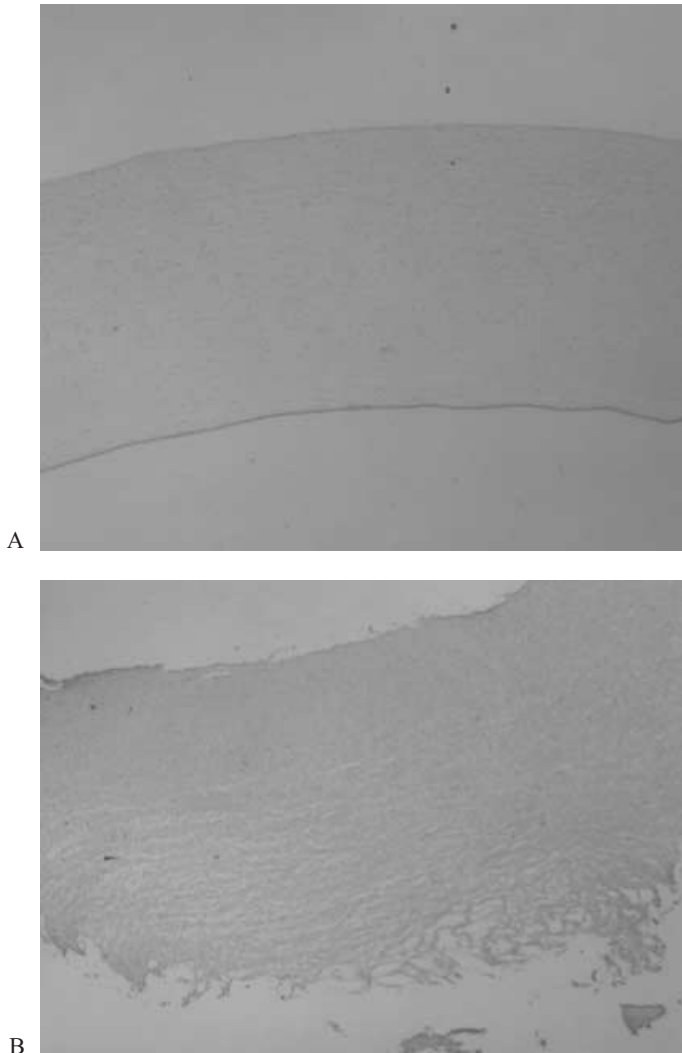
A

B

Figure 5. (A) Crosslinked control cornea without digestion. (B) Two-layer appearance of the treated cornea (3 mW/cm² on day 2) with loosely arranged, curly posterior collagen fibers and stiffened, densely packed anterior fibers in the crosslinked portion after collagenase treatment. Note complete absence of Descemet's membrane. (HE-stain, 200×)

Figure 6. Preserved anterior layer of the cornea crosslinked with riboflavin and UVA (3 mW/cm²) despite exposure to pepsin for five days.

most important mechanisms leading to corneal ulceration.[6,9,12,13] Tissue collagenases have been especially implicated in noninfectious corneal ulcers such as alkali-induced ulcer,[12] Mooren's ulcer, and rheumatoid ulcer[7,9] but also in infectious herpes-induced ulceration.[13]

The stabilizing biochemical effect by crosslinking can be explained by the changes of the tertiary structure of the collagen fibrils induced by crosslinking preventing access of the proteolytic enzymes to their specific cleavage sites by steric hindrance.[6] The efficacy of the treatment by metal-binding agents like EDTA or penicillamine in inhibiting collagenase activity is based on another principle, namely the fact that collagenases require metals for their enzymatic activity.[6]

In keratoconus, increased collagenase activity has also been found and may play a role in the pathogenesis of the
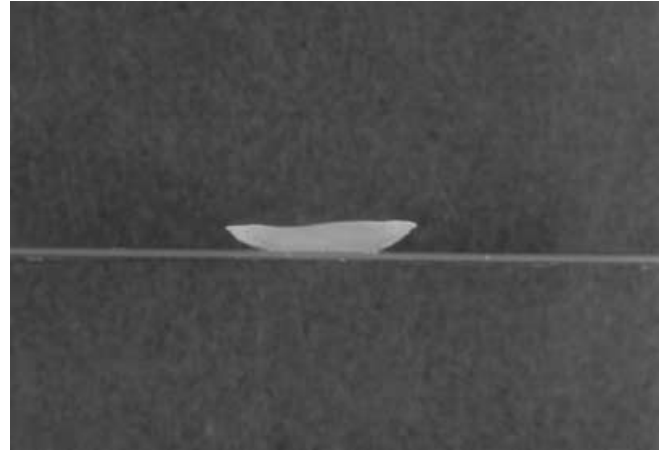
corneal thinning.[8] Accordingly, in tear fluid samples of keratoconus patients a 2.5 fold higher level of collagenase induced degradation products, so-called telopeptides, has been found.[14]

The protective effect of crosslinking against collagenase biodegradation is also used biotechnologically in the production of collagen-based implants such as amnion[15] or porcine heart valves.[16] Similarly, crosslinking of collagen implants from bovine corium with UVB resulted in a significant increase in resistance to pepsin,[17] trypsin and collagenase digestion.[18]

In the present study, we used *bacterial* collagenase as is usually done as part of a standard testing of the enzymatic resistance of biomaterials.[11] The bacterial collagenase is more potent and efficient than mammalian collagenase because it attacks multiple sites along the helix while mammalian collagenase cleaves the collagen helix at a single site across the three α-chains of collagen.[19] In addition, bacterial collagenase has a broader specificity spectrum digesting all types of collagen and ultimately reduces the collagen into small peptides and not only to a ¾ and 1/4-length product like human collagenase.[20,21]

By using electron microscopy to follow dissolution of the rabbit cornea, loss of parallel fiber array, leaving only few intact individual collagen fibers of the stroma, has been demonstrated previously by others after digestion with pepsin.[22] In our histological sections, we found similar changes in the posterior stroma where the degree of induced crosslinking is very low because of the up to 95% reduction of UVA-irradiance across the cornea by the massively riboflavin-enhanced UVA-absorption mainly in the anterior half of the cornea.[23] As the anterior stroma is essential for the maintenance of the anterior curvature of the cornea,[24] however, the localization of the crosslinking effect in the anterior stroma by the crosslinking method is an important asset because the corneal endothelium and the lens are spared from photooxidative damage.

Trypsin digestion for both crosslinked and non-crosslinked was only possible after prior heat-induced collagen degeneration. This is not surprising because trypsin is only capable of digesting at least partially denatured non-helical regions of collagen and therefore is sometimes used to assess the extent of denaturation of collagen.[25] The time for trypsin digestion after heating was markedly reduced compared to pepsin and collagenase presumably because the crosslinkages are partially destroyed already by the heat.

In summary, collagen-crosslinking in the cornea by riboflavin/UVA treatment significantly increases resistance to collagenase, pepsin and trypsin digestion especially in the anterior half of the cornea which is another important advantage of the new crosslinking method in addition to the increase in biomechanical stability following this procedure. This finding encourages the further use of this new method in the treatment of corneal ulcers and a prospective clinical study is currently underway.

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