

Technology of Local Cross-linking. Part 2: Experimental Results of Local Corneal Cross-linking

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ABSTRACT

Aim: The aim of the study was to assess the morphological changes in animal corneas following local cross-linking (CXL).

Materials and methods: Experimental comparative study included nine isolated porcine eyeballs (*ex vivo* group) and nine Chinchilla rabbits, one eye per animal (*in vivo* group). All the corneas in both groups were saturated with 0.1% riboflavin and corneal CXL was performed with Locolink device (Transkontakt, Russia), a system that allows to deliver ultraviolet (UV) radiation through a mask of any pattern. One half of each cornea was cross-linked and the other half remained intact. Three corneas of each group were irradiated according to the standard Dresden protocol with 3.0 mW/cm² for 30 minutes (5.4 J); yet, three corneas were irradiated in accelerated CXL mode I with 6.0 mW/cm² for 15 minutes (5.4 J), and yet, three corneas were treated in accelerated CXL mode II with 5.0 mW/cm² for 10 minutes (3.0 J). In the *ex vivo* group, the corneas of the whole isolated porcine eyeballs were examined with optical coherence tomography (OCT): The stromal demarcation line depth was evaluated by 10 different topographical points per image and the optical density of the corneal layers over the demarcation line was evaluated qualitatively as positive or negative. In the *in vivo* group, the rabbits were sacrificed 5 days after treatment, and their eyes were enucleated. Corneoscleral buttons were excised and subjected to histological and transmission electron microscopy (TEM) examination.

Results: *Ex vivo* group: Ultrastructural examination and OCT imaging showed that the demarcation line was more superficial after accelerated CXL than after standard CXL. *In vivo* group. No histological change was detected in the intact area. The re-epithelialization was complete by 72 hours after the procedure. Ultrastructural and histological examination on day 5 following complete re-epithelialization of the cornea showed the presence of bridge-like chains forming cross-links between

collagen fibrils in tangential sections. Keratocytes in irradiated zones were either in the state of apoptosis or were activated and produced newly formed collagen.

Conclusion: The method of local CXL allows to accurately and selectively modify limited regions of the cornea leaving other areas intact. In the case of local irradiation of the cornea, UV structural changes occur only within the irradiated area. The optimal protocol for use in local CXL is the Dresden protocol.

Keywords: Accelerated, Cross-linking, Local, Meta-optics, Personalized.

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INTRODUCTION

Corneal CXL, first developed in the early 2000s,¹ has since been improved over time and shown to be highly effective in keratoconus patients.²⁻⁶ Local (personalized) CXL is a modification of the standard CXL protocol that combines the reliability of the standard technology, short rehabilitation time, and the capability of more precise changes in corneal topographic characteristics.² In local CXL, not the whole cornea but its selected areas are irradiated with UV light in order to strengthen its weakest parts.

Local CXL is a novel technology and what changes it causes in the corneal stromal tissue are not yet fully understood. It is known though that after standard CXL procedure, corneal stroma is usually characterized by the presence of so-called demarcation line—the transition area between the cross-linked stromal collagen and intact tissue. Demarcation line can be visualized via OCT examination and is usually located in the middle of stroma of the cornea and is parallel to its surface. Another set of stromal tissue changes consists of structural and ultrastructural changes visible in optical and electron microscopy.

PURPOSE

To assess the morphological changes in animal corneas following local CXL.

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Table 1: Main parameters of CXL procedure used during CXL procedure in the experimental groups

Numbers of objects involved			Epithelium status	Intensity (mW)	Fluence total (J/cm ²)	UVA irradiation time (minutes)
Ex vivo group (isolated porcine eyeballs)	In vivo group (rabbit eyes)	CXL protocol				
3	3	Standard Dresden protocol	Off	3.0	5.4	30
3	3	Accelerated CXL mode I	Off	6.0	5.4	15
3	3	Accelerated CXL mode II	Off	5.0	3.0	10

MATERIALS AND METHODS

The ethical committee of the Eye Center East Sight Recover approved the research. All the experimental studies involving animals and animal tissue were conducted in accordance with Helsinki Declaration.

The current prospective experimental comparative study included two groups of experimental objects: Nine Chinchilla rabbits, one eye involved per animal (*in vivo* group) and nine isolated porcine eyeballs (*ex vivo* group).

Both Groups

Local CXL was performed with the use of the standard Dresden protocol² and two accelerated CXL protocols (Table 1). Briefly, corneal epithelium was debrided and then the cornea was saturated with 0.1% riboflavin (Pharmstandard, Ufa, Russia) for 30 minutes (Fig. 1). One half of each cornea was irradiated using a Locolink device which allows to deliver UV radiation through a mask of any specified pattern (Transkontakt, Russia). Three corneas of each group were irradiated with the use of the standard Dresden protocol with 3.0 mW/cm² for 30 minutes (5.4 J); other three corneas were irradiated in accelerated CXL mode I⁷⁸ with 6.0 mW/cm² for 15 minutes (5.4 J), and yet, three corneas were irradiated in accelerated CXL mode II with 5.0 mW/cm² for 10 minutes (3.0 J). During the CXL in isolated porcine eyeballs intraocular pressure of 20 mm Hg was maintained with the use of an irrigation system injected via the stump of the optic nerve. In all cases, non-irradiated area of the cornea served as



Fig. 1: Irradiation of one half of each rabbit cornea

an intact control. Table 1 summarizes main parameters used during CXL procedure in the experimental groups.

Ex vivo Group

Optical coherence tomography of anterior eye segment was performed in the whole isolated porcine eyeballs. The demarcation line depth was evaluated at 10 different topographical points per image and the optical density of the corneal layers above the demarcation line was assessed qualitatively as positive or negative.

In vivo Group

After the CXL procedure, all eyes were assessed with biomicroscopy. On day 5 postoperative, the rabbits were sacrificed, and their eyes were enucleated.

Corneoscleral buttons were excised out of the eyeballs and subjected to routine hemotoxylin and eosin staining (HES) tissue section and TEM examination.

RESULTS

Ex vivo Group

Ultrastructural examination and OCT imaging (Fig. 2) showed that the depth of the demarcation line was more superficial after accelerated CXL than after standard Dresden protocol. This finding indicates that the absorbed dose following accelerated CXL was higher due to the distribution of UV energy in the smaller volume of tissue.

Table 2 summarizes the main parameters evaluated in the nine porcine eyeballs.

The difference between demarcation line depths in three protocols was statistically significant ($p < 0.01$, analysis of variance F-test).

In vivo Group

During CXL procedure, riboflavin “burnout” was observed in the irradiated zone, but not in the intact zone

Table 2: Demarcation line depth and optical density data evaluated in isolated porcine eyeballs

CXL protocol	Demarcation line depth (μm) $M \pm \delta$	Optical density (+/-)
Standard Dresden protocol	290.0 \pm 9.1	+
Accelerated CXL Mode I	275.9 \pm 8.0	-
Accelerated CXL Mode II	241.3 \pm 9.1	+

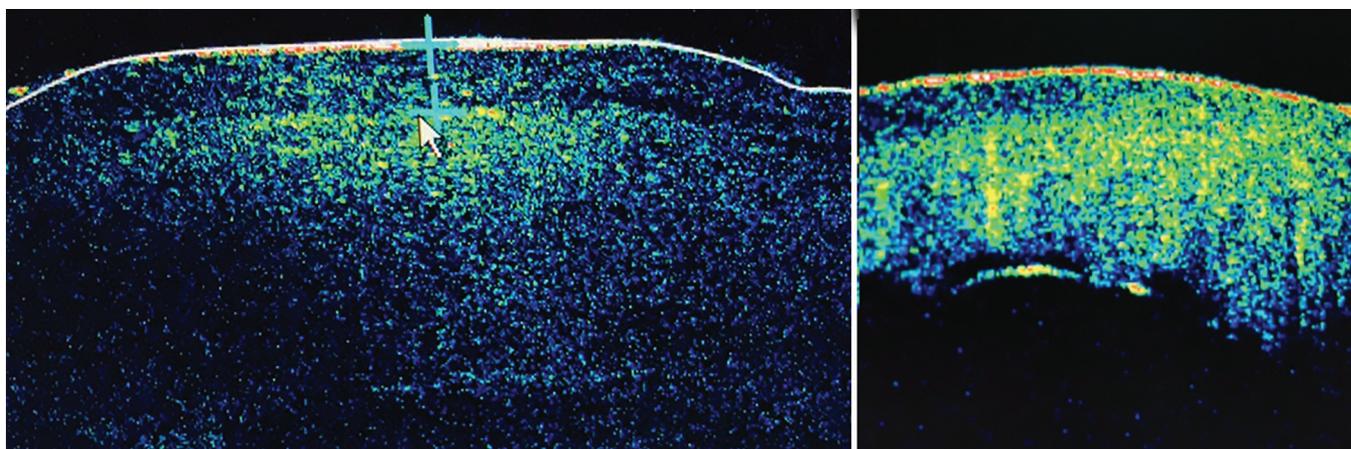


Fig. 2: The OCT and scanned images showing the negative reflecting area of an irradiated cornea. A demarcation line is present at a depth of 273 μm following irradiation at a power of 6 mW/cm^2 and an energy of 5.4 J (*left image*). Line of demarcation at the border of local irradiation (*right image*)



Fig. 3: Images showing a riboflavin "burnout" zone in the upper segment of the cornea after UV irradiation (*left image*) and a local haze zone in the upper segment of the cornea, corresponding to the UV irradiation zone, following the procedure (*right image*)

(Fig. 3). Corneal re-epithelialization completed 72 hours postoperative.

No histological change was detected in the intact zone in the HES tissue section examination in all cases. Ultrastructural TEM examination showed that the fibrous structure of collagen in the intact zone was retained (Fig. 4).

Ultrastructural and HES tissue section examination of irradiated areas on day 5 postoperative showed the presence of bridge-like chains that formed cross-links between collagen fibrils in tangential sections (Fig. 5).

Keratocytes in irradiated areas either were in the apoptotic state or were activated and producing newly formed collagen (Fig. 6).

Three spatial rearrangements of collagen fibrils were observed 5 days postoperative: compact fiber bundles (Fig. 7), compact fiber bundles with simultaneous defects of the fibril parallel orientation (Fig. 8), and ring-shaped collagen structures with end-to-end linkages (Fig. 9). The diameter of these structures ranged from 800 to 100 nm that is comparable to the wavelength range of visible light.

Examination of the rabbit corneas showed that when the radiation power exceeded 3.0 mW/cm^2 , even with an energy level remaining lower than 5.4 J, there was an

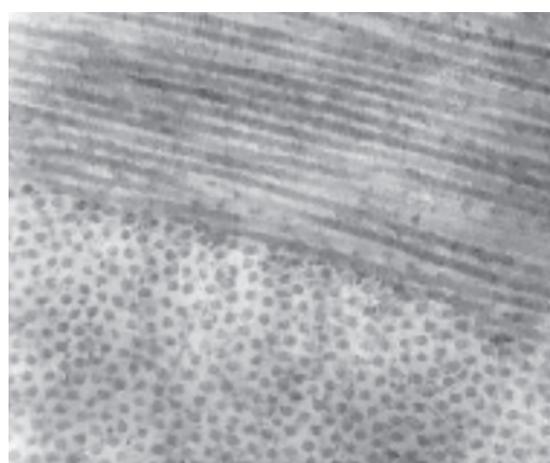


Fig. 4: The TEM image 5 days after removal of epithelium from the intact zone, showing no changes in the size or regular packing of collagen fibers ($\times 16,000$)

increase in the number of twisted collagen fibrils, along with the formation of annular structures.

DISCUSSION

The morphological findings detected in microscopic examination indicate that higher irradiation power intensified photodestruction processes in cornea during CXL. However, these ring-shaped structures (Fig. 9) may have meta-optic properties, substantially altering the refractive index of the tissue. This was indirectly confirmed by the appearance of a hyporeflecting zone after irradiation of *ex vivo* porcine corneas with maximum energy (Fig. 2). The presence of these meta-structures in corneas after CXL may explain, at least in part, the discrepancies observed between objective refractive control and the visual acuity in patients. Previously, those discrepancies were often ascribed to the inaccuracy of measuring devices.⁹ The combination of this optical effect with the concept of local CXL may enable the use of these treatment approaches for better control of corneal refraction.

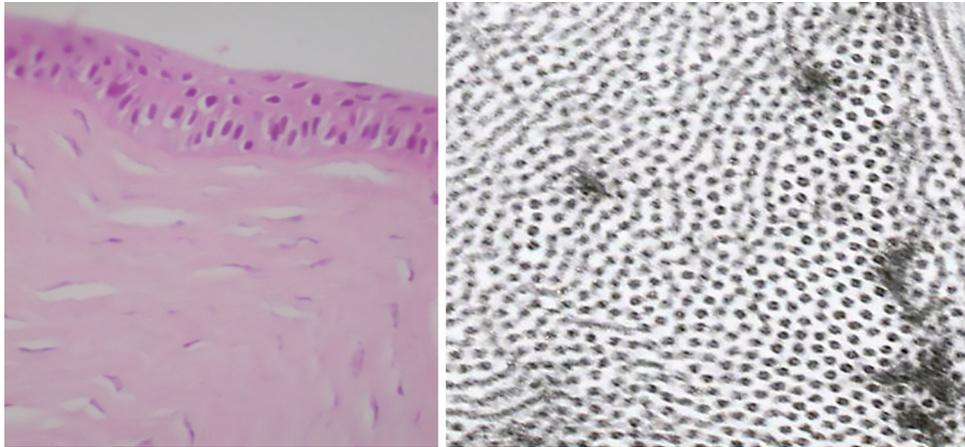


Fig. 5: Evaluation of the irradiated area of the cornea 5 days after the procedure. Histologic examination showing complete re-epithelialization (HES tissue section $\times 240$; *left image*). TEM examination showing chain-like cross-links between collagen fibers in tangential areas ($\times 18,000$; *right image*)

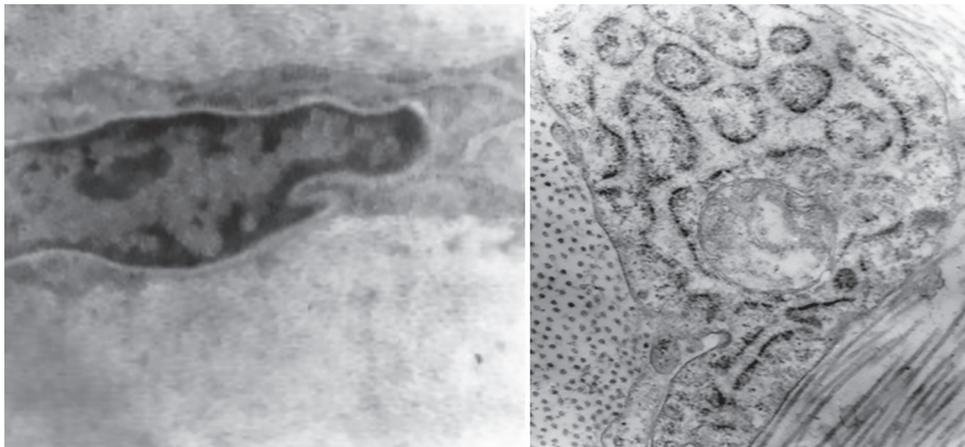


Fig. 6: The TEM images of the irradiated zone showing keratocytes in the state of apoptosis ($\times 8000$, *left image*) and activated fibroblasts producing collagen ($\times 16,000$, *right image*)

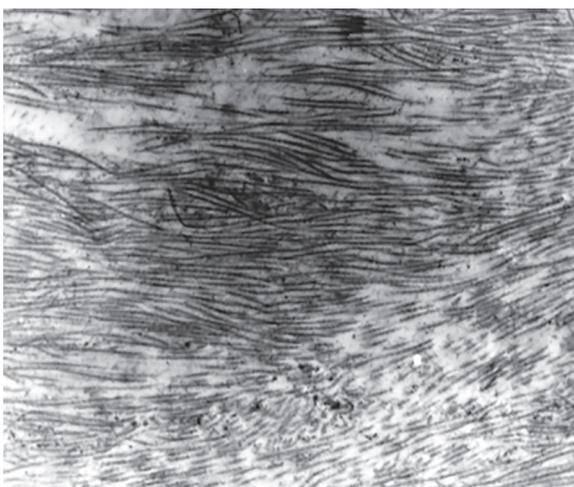


Fig. 7: The TEM image showing compact collagen fibrils 5 days after UV irradiation ($\times 10,000$)



Fig. 8: The TEM image showing compact collagen fibrils with simultaneous defects of the regular fibril orientation 5 days after UV irradiation ($\times 18,000$)



Fig. 9: The TEM image showing the formation of ring-shaped collagen structures in place of regular parallel collagen bundles 5 days after UV irradiation ($\times 16,000$)

CONCLUSION

The method of local CXL allows to accurately and selectively modify the specified areas of the cornea leaving other areas intact. In the case of local irradiation of the cornea, UV structural changes occur only within the irradiated area. The optimal protocol for use in local CXL is the Dresden protocol.

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