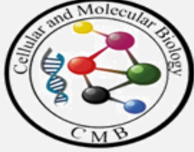

Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

C M B Association Publisher

Evaluation of corneal tissue changes after collagen cross-linking with ultraviolet and riboflavin A

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ARTICLE INFO

Original paper

Article history:
Received: January 06, 2022
Accepted: March 15, 2022
Published: May 31, 2022

Keywords:
Corneal Tissue, Collagen Cross-Linking, Eye, Keratoconus

ABSTRACT

This study was performed to evaluate the histological changes in the cornea after accelerated collagen crosslinking. In this regard, the right eyes of 7 New Zealand albino rabbits weighing 1.5 to 2 kg were studied. The right eye was considered the case group, and the left eye was considered the control group. The right eye epithelium was removed, riboflavin 0.1 solutions (10 mg riboflavin-5 phosphate in 10 ml dextran-T-500, 20%) were shaken every three minutes for 30 minutes and exposed to UVA for 10 minutes, and crosslinking was performed. All rabbits were euthanized and histologically evaluated. Apoptosis was assessed using the tunnel method. The results showed a significant difference in the mean percentage of apoptotic cells in the treatment and control groups. In both endothelial cells and keratocytes in the treated cornea, the number of apoptotic cells was significantly higher than in the control group. In the general examination of the cornea (keratocytes and endothelium) in the right eye of rabbits, the mean and standard deviation of the percentage of apoptotic cells was 18.39 ± 3.4 and in the left eye was 6.37 ± 1.8. The apoptosis results of keratocytes in the right eye showed that the mean and standard deviation for the percentage of apoptotic keratocytes was 2.86 ± 1.07, which was significantly higher than in the left eye (0.53 ± 0.43) (P <0.001). The mean and standard deviation of endothelial apoptotic cells in the right and left eyes were 86.2 and 53.5, respectively, statistically significant (P = 0.026). Although CXL is a reliable method of preventing the progression of keratoconus disease, there is still a risk of endothelial cell damage and related complications due to endothelial cell damage, and further studies are needed.

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Introduction

Keratoconus is a bilateral, non-inflammatory, progressive thinning, and progressive corneal instability that presents with abnormal thinning, increased curvature, scarring, and protrusion of the central part of the cornea (1). This increase in curvature causes a change in refractive power, followed by irregular myopia and astigmatism, which results in a slight to a significant reduction in visual quality (2). The prevalence of keratoconus is 200-50 per 100,000 people during puberty (3). The treatments for keratoconus are first glasses, then hard contact lenses, corneal intra-stromal rings, and corneal transplants. This disease is the leading cause of corneal transplantation in developed countries (1).

Unfortunately, all treatments for corneal ectasia are focused solely on correcting refractive errors, which do not treat the underlying cause of ectasia and cannot prevent the development of keratoconus (2). In recent decades, collagen crosslinking has become commonplace to prevent disease progression (4).

Collagen Crosslinking (CXL) induces crosslinking within and between collagen fibers and increases biochemical strength in the human cornea by approximately 300% (5). The maximum crosslinking rate is in the anterior stroma. Research on collagen crosslinking is ongoing to determine if CXL can slow or stop the progression of corneal ectasia in patients with keratoconus or reverse the course of the disease (4).

There have been many studies on this method's side effects and complications (5, 6). Still, despite many studies on the safety of this method, many side effects and reports of corneal endothelium damage have also been reported (3). *In vitro* studies have shown that approximately 30% of UVA light (ultraviolet A) is absorbed by the cornea, while UVA absorption by the lens is 50% higher. With an intensity of 3mW/cm^2 in UVA and 0.1% riboflavin, a maximum of 95% ultraviolet light will be absorbed in the cornea (7). As

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a result, small amounts of UVA can penetrate the eye (8). This study aimed to evaluate the histological changes of corneal histology after CXL accelerated in experimental studies of rabbits.

Materials and methods

Seven New Zealand white adult male rabbits were used. The rabbits were all healthy. This study was approved by the Ethics Committee for Animal Experiences and was based on the ARVO Statement on the Use of Animals in Ophthalmology. General anesthesia was treated with ketamine hydrochloride 35 mg/kg and xylazine hydrochloride 5 mg/kg. The central epithelium in the right eye was removed with 20% ethanol, and the left eye remained intact as a control group. For the right eye of rabbits, riboflavin drops of 0.1% (10 mg riboflavin 5-phosphate in 10 ml dextran 500-T) which crosslinking was performed once for 30 minutes at 3-minute intervals. A 370nm dual diode performed ultraviolet irradiation with an irradiance of $3mW/cm^2$ for 10 minutes (final dose 4.5 J/cm^2). Riboflavin drops were instilled every 3 minutes during UV irradiation. Immediately after the operation, chloramphenicol and betamethasone drops were instilled in both rabbit eyes and then one drop every 6 hours for 24 hours.

Tissue preparation

Rabbit eyes were fixed in 10% neutral formalin 24 hours after ingestion. After a 150 mg/kg pentobarbital injection, the rabbits then died quickly and painlessly.

Tunnel Assay

In this study, tunnel staining was used to measure the number of apoptotic cells. Fragmentation is an irreversible reversible process that occurs even before changes in membrane permeability occur. Calcium- and magnesium-dependent activation of nuclear endonuclease appears to cause this event. This enzyme selectively breaks DNA locally between nucleosome units. It is examined by tagging an enzyme through the tunnel that can identify the ends of defects in the DNA strand. In this study, the samples were stained with the help of Roche (Situ Cell Death Detection Kit POD; Germany). Thus, the samples were placed in xylol for 10 minutes after paraffin removal. The slides were then watered at 90°C, 80°C, and 70°C. The lamellae were then washed using PBS solution and incubated in proteinase k for 20 minutes at 37°C. Then, the tissue slides were incubated with the absorbent solution for 10 minutes and washed again with PBS. In the next step, 50µl of tunnel dye solution was poured into each tissue sample and incubated for one hour at 37°C after final washing with a fluorescent microscope (Zeiss LSM 5 fluorescent microscope). Five fields were counted in each group to count dead cells. An area of 1 square mm in each group was considered for counting. In this protocol, for the staining of positive control samples, both TUNEL-Enzyme solution and TUNEL-Label solutions were used in a 1: 9. While for negative control samples, only TUNEL-Label solution was used. The apoptotic cells in this tissue were bright spots that represented the apoptotic cells labeled during tunnel staining.

Statistical analysis

To evaluate apoptosis, first, the mean and standard deviation of the percentage of apoptotic cells in the whole right corneas of the right eye (case group) and left eye (control group) were measured. Endothelial and total corneas were calculated. The normality of data distribution was assessed using Kolmogorov-Smirnov and Shapiro–Wilk tests. Wilcoxon matched-pairs signed-rank test (2 samples) was used to compare the mean and standard deviation of the percentage of apoptotic endothelial cells in the right and left eyes that did not have a normal distribution. Paired t-test was used for apoptosis is normally distributed in keratocytes and corneas.

Results and discussion

The results of tunnel assay showed that tunnel-positive cells (apoptotic cells) were observed in crosslinked areas in endothelium and keratocytes (Figure 1). There was a significant difference in the percentage of apoptotic cells between the treated and control eyes in all samples. The mean rate of apoptotic keratocytes in the treated eye was significantly higher than the control group in all animals. Also, the mean percentage of apoptotic cells in endothelial cells was significantly higher than in the control group.

In the general examination of the cornea (keratocytes and endothelium) in the right eye of rabbits, the mean and standard deviation of the percentage of apoptotic cells was 18.39 ± 3.4 and in

the left eye was equal to 6.37 ± 1.8 (P <0.001) (Figure 2).

In the apoptosis study of keratocytes, the mean and standard deviation of apoptotic keratocytes in the right eye was 15.27 ± 2.46, and in the left eye (5.65 ±1.4) was significantly higher (P <0.001) (Figure 3).

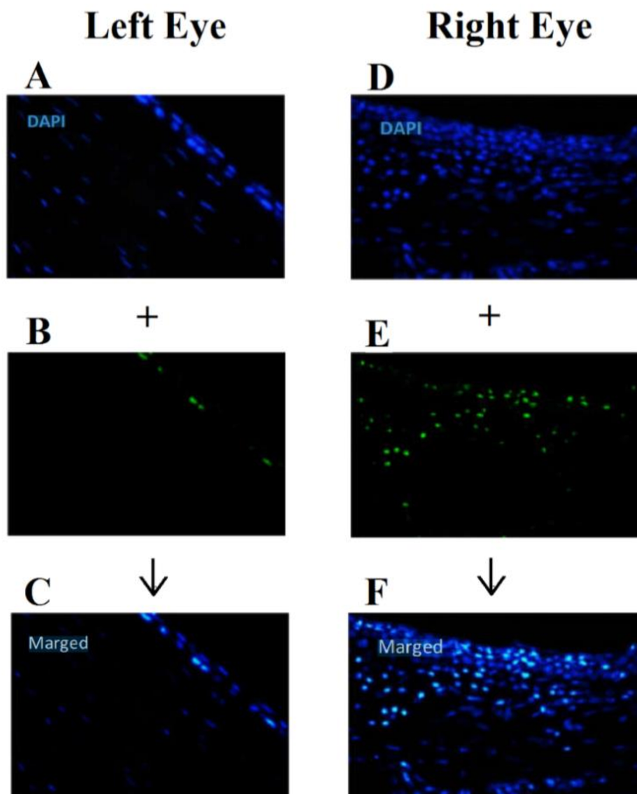

Figure 1. The results of tunnel assay in both eyes of sample No.6; Left eye belongs to control group and right eye belongs to intervention group. "A" and "D" are the nuclei of keratocytes and endothelium, which are shown in blue. "B" and "E" are apoptotic cells, which are shown in green. "C" and "F" are produced by merging the two above images, in which all the apoptotic cells are seen in glossy blue.

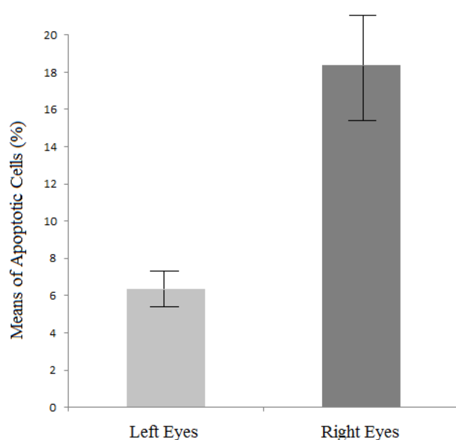

Figure 2. Mean percentage of apoptotic cells in cornea

The mean and standard deviation of endothelial apoptotic cells in the right and left eyes were 2.86 ± 1.07 and 0.53 ± 0.43, respectively. Comparing these two groups with the nonparametric Wilcoxon Signed Ranks Test, the difference between the two groups was statistically significant (P = 0.026).

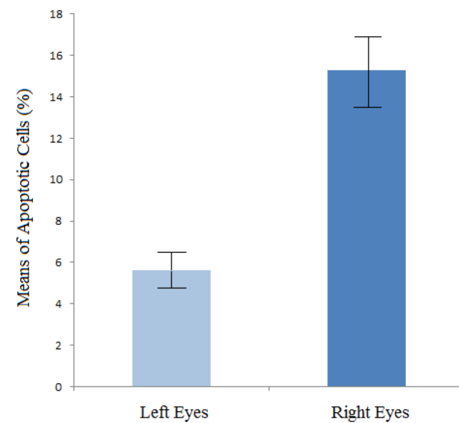

Figure 3. Mean percentage of apoptotic cells in keratocytes

Collagen crosslinking in the cornea is almost a non-invasive process that increases the stiffness of the cornea and subsequently delays and stops the progression of the disease (8). As the riboflavin is exposed to radiation, it becomes ternary and releases reactive oxygen species. Oxygen is released, and superoxide ions react with close and available groups (9). One of the possible mechanisms of CXL seems to be the formation of chemical bonds between histidine, hydroxyproline, hydroxylysine, tyrosine, and threonine amino acids (8). UVA absorption in corneal stromal layers has been proven to be approximately 30%, while in combination with riboflavin photomodulator, this absorption increases from 30% to 90% (10).

We performed this study to evaluate the effects of CXL on corneal stromal cells in humans. Previous *in vitro* studies have shown that combination therapy with riboflavin and UVA radiation results in a keratocyte cytotoxicity threshold ten times lower than UVA alone (11). Our study showed that collagen crosslinking causes premature edema and extensive apoptosis of keratocytes, and increased corneal thickness and endothelial apoptosis at 3mW/cm^2 surface radiation. Also, the mean percentage of endothelial apoptotic cells was lower than the mean percentage of apoptotic cells of keratocytes, which is

related to the deeper position of endothelial cells that received less UVA radiation.

Wollensak *et al*. (12) showed that in rabbit corneas less than 400µm thick, the level of UVA cytotoxicity reached 0.65 J/cm^2 for endothelial cells using radiation at 4.5 J/cm^2. In our study, the rabbit cornea was less than 400µm thick, and like Wollensak *et al*. study, we observed apoptosis in all endothelial and stromal layers. In several previous studies, keratocyte loss occurred only at a thickness of 250–300µm in the stroma, which was inconsistent with our and Wollensak *et al*. study, which showed apoptosis in all layers of the cornea. Recently, endothelial cell damage in the human cornea after CXL has been reported to indicate endothelial cytotoxicity even in the thick cornea. Sharma *et al*. (13) reported corneal edema in 10 of 350 patients who underwent CXL surgery, two of whom eventually developed perforated keratoplasty.

In our study, the increase in apoptotic endothelial cells in the treatment group was similar to the Sharma report, which indicates an increased risk of endothelial cell damage after CXL. In the study of Salomon *et al*. (14), the histopathology of rabbit cornea after CXL, premature edema and apoptosis, and keratocyte necrosis occurred to 250µm. Also, inflammatory cells in the cornea and the decay of keratocytes to myofibroblasts were observed from the third day to the next day. Like the Salomon et al. study, primary edema and keratocyte necrosis also occurred in our study. Still, unlike their study, apoptosis also occurred in the entire depth of the stroma and even endothelium. On the other hand, in the study of Salomon *et al*., there was the infiltration of inflammatory cells and deformation of myofibroblasts, while in our study, these cases were not present. It may be because the inoculation was performed in our study in the first 24 hours, and there was not enough opportunity for infiltration and deformation.

In several studies, endothelial cell depletion after CXL has been reported by both traditional and accelerated methods (15-17). For example, Badawi (15) showed endothelial cell reduction after accelerated CXL with 9 minutes and 10mW/cm^2. In Sadoughi *et al*., (16) a decrease in endothelial cells was observed in traditional CXL patients, not significant. In a study by Hovakimyan *et al*. (17) performed on rabbits, keratocytes were lost throughout the stroma, and confocal scan and immunohistochemistry demonstrated significant endothelial cell death. However, in this study, complete repair of the endothelial layer of the rabbit cornea was observed one week after CXL. Mitosis was also observed in endothelial cells, indicating the strength of corneal endothelial repair in rabbits. As reported in many animal studies, apoptosis in corneal endothelium was present in our analysis. Reports of endothelial damage in the human cornea greater than 400µm suggest that CXL can still have side effects and require further investigations.

Conclusions

Although CXL is a reliable way to prevent the progression of keratoconus disease, it is still dangerous due to endothelial cell damage that occurs even in the human cornea and more studies are needed to evaluate the side effects of this method.

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

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