acta medica

ORIGINAL ARTICLE

Enzymatic Digestion of Fresh-Frozen Human Cornea After Riboflavin/Ultraviolet-A Collagen Crosslinking

Ayşegül Fırat[1]
ORCID: 0000-0001-5105-0057

Züleyha Yalnız Akkaya[2]
ORCID: 0000-0003-3817-2214

Özlem Dikmetaş[3]
ORCID: 0000-0001-5670-2384

Hatice Mürvet Hayran[4]
ORCID: 0000-0001-6058-6304

[1]Department of Anatomy, Hacettepe University Faculty of Medicine, Ankara, Turkey.

[2]Department of Ophthalmology, University of Health Sciences, Ankara Training and Research Hospital, Ankara, Turkey.

[3]Department of Ophthalmology, Hacettepe University Faculty of Medicine, Ankara, Turkey.

[4]Department of Anatomy, Economy University Faculty of Medicine, Izmir, Turkey.

Corresponding Author: Ayşegül Fırat
Department of Anatomy, Hacettepe University Faculty of Medicine, Ankara, Turkey.
E-mail: aysfirat@hacettepe.edu.tr

Received: 11 January 2022, Accepted: 11 May 2022, Published online: 13 June 2022

ABSTRACT

Objective: Crosslinking (CXL) of the cornea by using riboflavin (RBF)/ ultraviolet-A (UVA) has been developed for stiffening the collagenous matrix of the cornea. Collagenases and other metalloproteinases are known to play an important role in the pathogenesis of corneal ulceration. Our aim in this study was to show and compare the morphological and histopathological changes of the CXL human corneas against enzymatic digestion by collagenase, trypsin and pepsin solutions.

Materials and Methods: Eighteen corneas from nine fresh frozen cadavers were included in this study. The crosslinked group (n=12) was treated with the combined riboflavin (RBF) and ultraviolet-A (UVA) and the control group (n=6) was only treated with RBF. After treatment corneas were exposed to collagenase, trypsin and pepsin enzymes. For each enzyme group one cornea was evaluated morphologically and the other one was evaluated microscopically.

Results: The collagenase treated control group, digestion of the cornea was noted in the first days and completed on the 5th day. The control cornea treated with pepsin was fully digested after 14 days. In the trypsin treated CXL cornea complete digestion was noted after 18 days. In both groups, enzyme activity was observed to be parallel to each other morphologically, but CXL cornea was evaluated to be more resistant to enzymatic digestion.

Conclusion: In conclusion, CXL increases resistance of the cornea against enzymatic digestion. In addition to the biomechanical support, this study was also showing the histopathological changes of CXL procedure and the results of enzymatic digestion, supporting new treatment options in the corneal diseases.

Keywords: Corneal crosslinking, collagenase, pepsin, trypsin

INTRODUCTION

Corneal crosslinking (CXL) causes an increase in the biomechanical strength of cornea by increasing collagen fibril diameter and stiffening the collagenous matrix, leading to an additional band of intense polymer in electrophoresis [1]. Enzymatic digestion is an indicator of biomechanical support of CXL cornea. Relevantly, CXL porcine corneas have been demonstrated to be resistant to collagenase, trypsin and pepsin digestion [2-4]. Since collagenases play an important role in corneal ulceration in different conditions such as infective keratitis, chemical burns or peripheral ulcerative keratitis; increasing the resistance of the cornea against the effect of these enzymes can be achieved by CXL [5-7]. Thus, in this study we aimed to evaluate the resistance of CXL human cadaveric cornea against enzymatic digestion.

© 2022 Acta Medica.

MATERIALS AND METHODS

The study was carried out in the Department of Anatomy, Hacettepe University Faculty of Medicine. In this study 18 corneas of nine fresh-frozen human cadavers were used. Six of the corneas were reserved for the control group and 12 of them were included in the CXL group. Before the corneas were trepanized, a deep-lysed riboflavin (RBF) solution was applied to the cornea (3-4 drops every 2 minutes) for 20 minutes in the control group. In the CXL group same procedure (3-4 drops of Rbf every 2 minutes) was followed by UVA irradiation (CBM Vega X-Link CSO srl, Scandicci, Firenze, Italy) 1 cm away from the cornea at 370 nm wavelength (3 mw / cm2) for 30 minutes. During irradiation, RBF was dropped every 5 minutes to increase the sensitivity of the cornea to the beam. The corneas were then trepanized with an 8.25 mm trepan and immediately placed into seperate glass containers with collagenase, trypsin and pepsin enzyme solutions. One sample of each group was selected for morphometric evaluation and others were prepared for light microscopic examination. Among six corneas included in the control group, selected two were treated for each enzyme: one cornea was photographed for morphometric changes (diameter, slope, digestion and transperancy) in the first, third and fifth days of the experiment; the other one was divided into three equal parts for enzymatic treatment. For each of the enzyme treated corneas in the CXL group; one cornea was photographed for morphometric parameters and others were evaluated microscopically in the first, third and fifth days. Collagenase-A (0.1 mg / ml colA) solution was made up of 7.9 mg of collagenase (Roche, Mannheim, Germany, EC 3.4.24.3) in 20 ml of phosphate buffer solution (PBS). Working pepsin solution (0.4%) was prepared by 80 mg of purified pepsin (Sigma, Munich, Germany, EC 3.4.23.1) in 20 ml of 10 mM HCl at optimum pH=1.5 for pepsin activity. Trypsin working solution with a 0.125% trypsin (Thermo scientific, Cheshire, UK, TA-015-TR) was made up of 1:3 trypsin in PBS buffer (5 mlt 0.125% trypsin+15 mlt PBS). Before incubation with trypsin, protein heat denaturation was performed by boiling the cornea in distilled water at 100 degrees for 10 minutes [2]. Then the corneas were taken into glass tubes and the enzymes were added immediately at room temperature. Slope and diameter measurements were made by using a millimetric paper. In order to measure the slope, dome of the cornea was placed on the slide and the height of the corneal edge from the slide was measured (Fig 1). A four grade scoring scale was performed for transparency and digestion (Four points given for the most transparent and intact corneas, zero point for the least transparent and digested corneas).

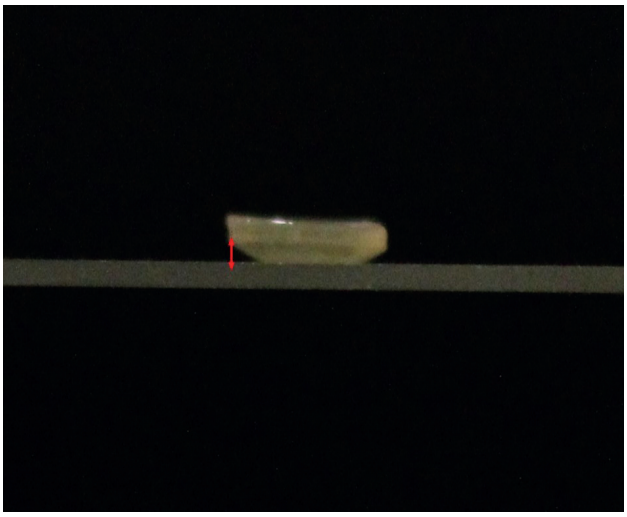


Figure 1. Slope of the CXL cornea after two days of digestion.

For histological evaluation, samples in different enzyme groups were fixed in 2.5% gluteraldehyde (Agar Scientific Ltd., Essex, UK, R1010) for 24 h, at room temperature. Then, samples were post-fixed in 1% osmium tetroxide (Millipore Sigma, Burlington, MA, USA, 56H1140) at 4 °C for 2 h. Following post-fixation tissue samples were dehydrated and then embedded in epoxy resin (Araldite CY212 kit, Agar Scientific Ltd., Essex, UK, AGR 1030). After plastic embedding, approximately 4 µm thick semi-thin sections were cut with a glass knife on an ultramicrotome (LKB Nova Ultramicrotome, Bromma, Sweden). Semi-thin sections were stained with 1% methylene blue (BDH Ltd., Poole, UK, CAS 61-73-4) solution (methylene blue 1 gr + sodium borate 1 g + distilled water 100 ml). Stained sections were examined and captured under the camera Lucida of a Nikon Optiphot (Nikon Corporation) light microscope for histologic analysis (Fig 2).

© 2022 Acta Medica.

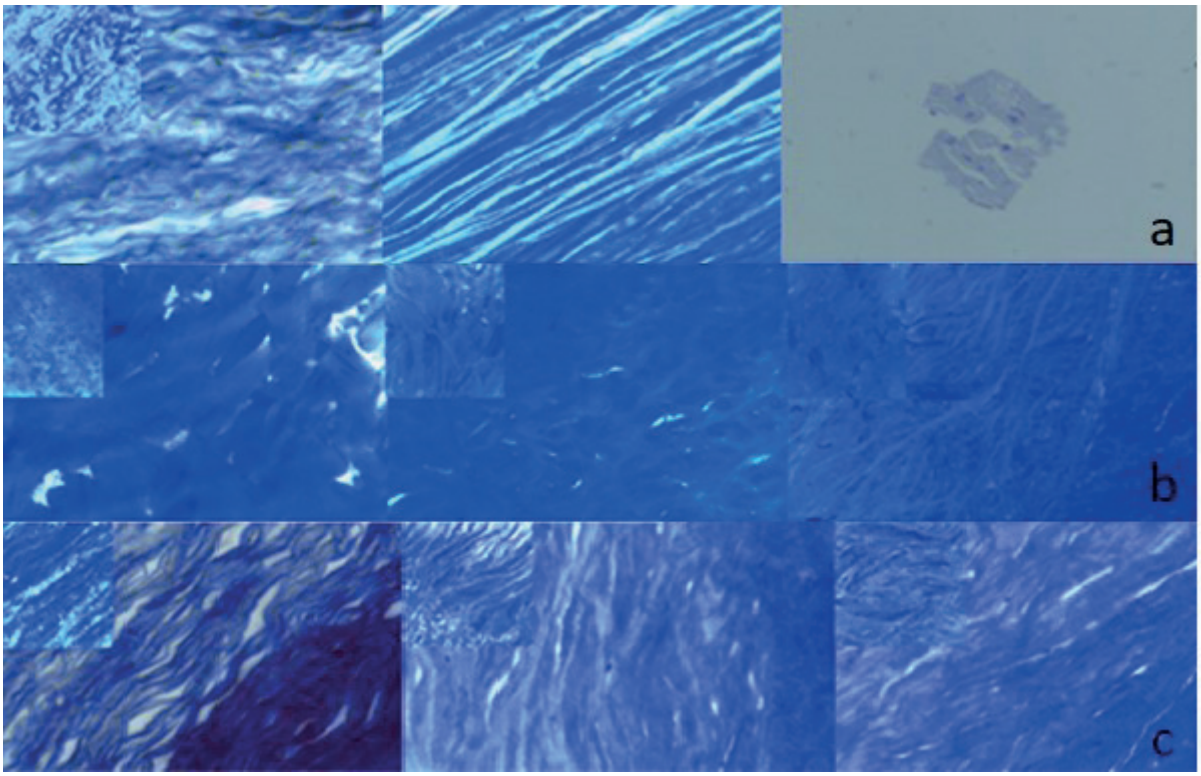


Figure 2. Microscopic evaluation (1st, 3rd and 5th days respectively), a. Corneas treated with collagenase and its control groups on the inset, b. Corneas treated with trypsin and its control groups on the inset, c. Corneas treated with pepsin and its control groups on the inset. Methylene blue staining, x40.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards and approved by the local Ethics Committee (GO 13/275-34).

Statistical analysis

In our study, measurements were made for a total of six observations and two observations (control-CXL) were performed with the same enzyme by a blind observer (AF). We were not able to perform hypothesis tests for comparing enzyme and treatment effects because the sample size was very small. Therefore, statistical evaluation was performed using only the descriptive statistics, and tables and graphs were used instead of hypothesis testing. During follow-up, means were used for morphometric descriptions. In particular, line graphs of first, third and fifth days were considered important for morphologic changes over time and for comparisons with histologic findings (Fig 3,4).

RESULTS

In the collagenase treated group, digestion of the control cornea started immediately and the sample was fully digested on the fifth day. The collagenase treated CXL cornea was also digested rapidly and digestion was completed on the sixth day. The CXL cornea was slightly opaque during the first days of the follow-up, then it became transparent following digestion. Its slope was zeroed in parallel with digestion and its diameter was increased during a process like melting. In both groups, the effects were parallel, but CXL cornea was observed to be more resistant to digestion. Both corneas completed the digestion process not with disintegration but with melting, also without a morphometrical changes were apperent in the transparency (Fig 5).

The control cornea treated with pepsin was fully digested in the 14th day. During the follow-up, the transparency was decreased by half, the slope and the diameter was decreased during the first seven days. After seventh day, the diameter was increased

© 2022 Acta Medica.

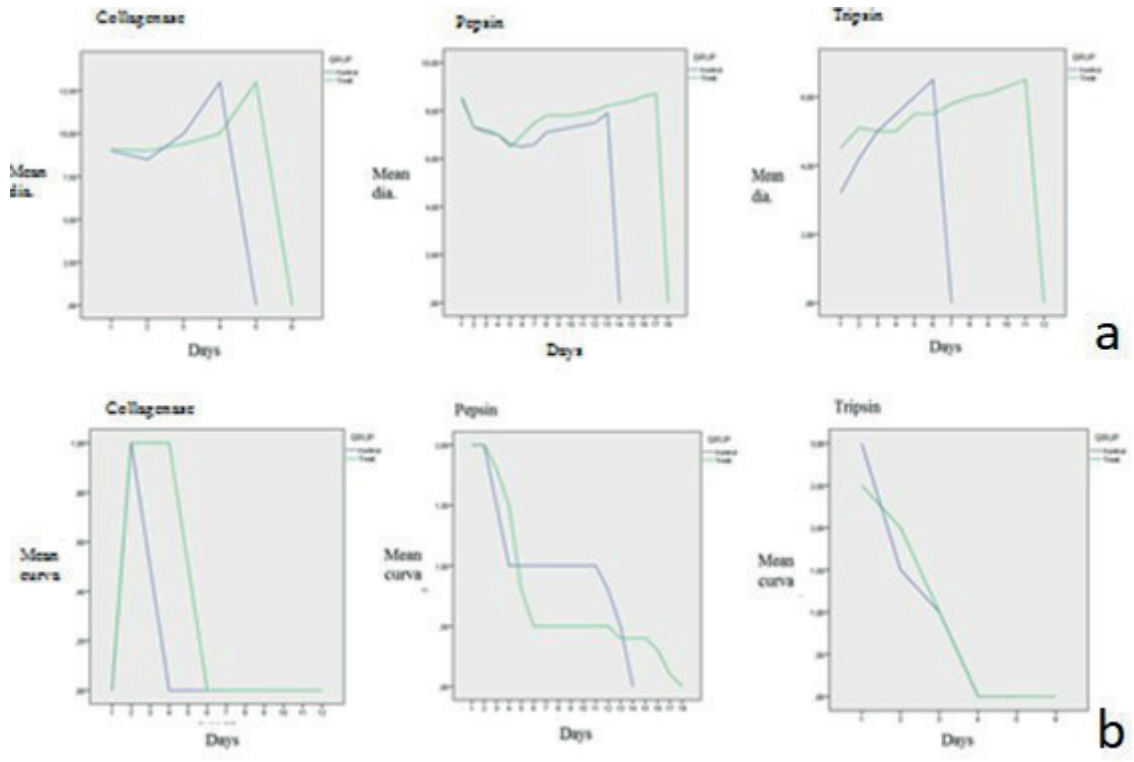


Figure 3. Graphics showing daily changes of a. diameter and b. curvature in control and CXL groups.

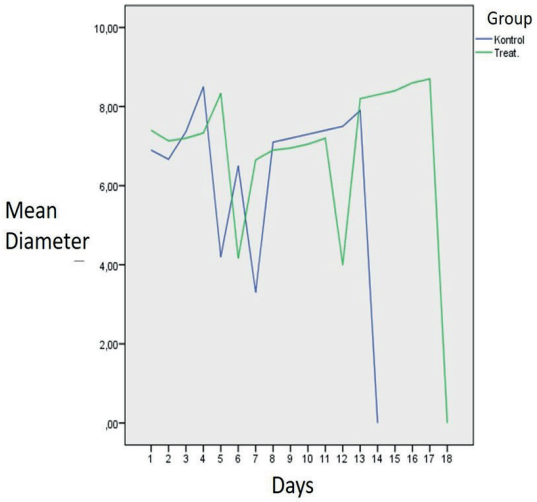


Figure 4. Graphic of the mean diameter change of corneas over time.

because of digestion with melting. The CXL cornea, treated with pepsin was completely digested after 18 days. The diameter of CXL cornea was decreased until the 6th day, then began to increase. Slope of the CXL cornea was decreased throughout the digestion process. In both groups, enzyme activity was observed to be parallel to each other, but CXL cornea was resistant to digestion same as in the collagen treated group. Both corneas were shattered with reduced transparency and completed enzymatic digestion (Fig 6).



Figure 5. Collagenase treated control group (2nd day).



Figure 6. Pepsin treated control group (left) and CXL group (right) (2nd day).

© 2022 Acta Medica.

Since the corneas treated with trypsin enzyme were first denaturated by heat, their diameters were reduced by half and their transparency was slightly transparent at the onset of the procedure. The control cornea was disintegrated after six days and complete digestion was noted on the seventh day. In the follow-up of the control cornea, it was observed that its transparency and diameter increased as it approached the day of digestion, and the slope was increased at the beginning and then decreased after the second day of digestion procedure. The CXL cornea treated with trypsin was disintegrated on the eighth day and complete digestion was observed on the 12th day. During the follow-up, it was observed that the initially increased opacity decreased with the digestion process. The slope was increased during the first four days and then decreased and the diameter was decreased regularly throughout digestion. Both corneas were disintegrated into pieces without full transparency and the digestive effects were completed in parallel.

Light microscopic images were also evaluated for the first, third and fifth day and these evaluations were supported by the morphometric results.

The histological evaluation of the control group treated with collagenase was performed only for the first day as digestion and melting was developed very rapidly. On the first day, collagenase impaired the lamellar appearance of the cornea in the control group and the gaps between the fibers were remarkable. On the same day, the lamellar appearance was preserved in the CXL cornea but gaps were observed in some areas. On the third day gaps were increased in number without a disruption on the lamellar organization. A very soft and gelatinous sample was prepared for microscopic evaluation after five days and collagen fibers were observed in these sections. On the first day, the convoluted appearance of the fibers in the cornea was also consistent with the slight decrease in the diameter. During the follow up as cornea became more gelatinous, the diameter and convolutions were both increased.

No gradual increase was observed in the lamellar appearance of collagen in the control and CXL corneas treated with trypsin. The morphology was consistent with the findings in diameter and slope graphs. Until the end of fifth day, uniform lamellar appearance and lack of gaps between fibers were remained almost similar for both groups.

Since the digestive effect of pepsin was noted as the latest, there was no gradual difference in lamellar appearance in the control and CXL corneas for the first days of digestion.

DISCUSSION

Corneal melting occurs in various infectious and non-infectious inflammatory conditions that threaten the vision and the integrity of the eye. It has been shown that the corneal melting is caused by various digestive enzymes [5,7-9]. Thus, in order to save the eye and the vision, the corneal melting must be prevented. This melting processes can be halted by two mechanisms: by decreasing the production of degrading enzymes or by increasing the strength of the cornea to the enzymatic digestion. The later effect is achieved by combined Riboflavin/UVA CXL of the corneal stroma [10-13]. Thus, the corneal stroma becomes more resistant to digestive enzymes from microbial and inflammatory origin [14]. The structural and biomechanical effects of Riboflavin/UVA CXL in the corneal stroma have been evaluated both clinically and experimentally [10-13,15,16]. For in vitro evaluations light, electron and confocal microscopy, x-ray scattering and second harmonic generation imaging, 2-dimensional fast Fourier-transform analysis stress-strain biomaterial tester and enzymatic digestion have been used [17]. The stiffening effect of Riboflavin/UVA CXL on the normal human, rabbit and porcine corneas have been measured by stress-strain biomaterial testers [18-20]. For the first time, Wollensak et al. demonstrated increase in corneal rigidity by 328.9% in 5 human cadaveric corneas, while the increase in 20 porcine corneas was by 71.9% [20]. This biomechanical effect was explained by the increase of 12.2% in collagen fiber diameter due to interfibrillary cross links in rabbit anterior cornea [1]. While 22.6% increase was observed in anterior healthy human cornea, no increase was noted in posterior cornea. In bovine corneas the adherence force (measured with extensometer) of laser in-situ keratomileusis (LASIK) flap after CXL has been

© 2022 Acta Medica.

shown to increase and gradually decrease in organ culture afterwards. The authors were not been able to explain this transient effect. The strengthening effect has been shown to occur due to intrafibrillar and interfibrillar crosslinking but not interlamellar [21]. After CXL, a strong band of high molecular-weight collagen polymers that was resistant to mercaptoethanol, heat, and pepsin treatment has been detected by electrophoresis in porcine corneas [3]. As a result of these structural changes, crosslinked porcine and rabbit corneas were found to be more resistant to pepsin digestion. Kanellopoulos et al. evaluated the biomechanical and enzymatic digestion resistance differences between LASIK and LASIK+CXL. The latter showed significant increase in underlying corneal stromal rigidity [22]. Alageel et al. analyzed whether verteporfin with a nonthermal laser increases corneal mechanical stiffness and resistance to enzymatic degradation ex vivo. Corneal resistance to enzymatic collagenase degradation.was notably increased with verteporfin [23]. For the first time Wollensak et al. demonstrated that CXL corneas were dissolved by day 13 and 14 following pepsin and collagenase treatment versus six days in control group in porcine corneas. After heat denaturation, digestion by trypsin was observed by day five and day two respectively in CXL and non-CXL corneas [1]. The anterior curvature was maintained in the first seven days in pepsin and collagenase treated group. A study with all non-CXL corneas (8.5 mm in diameter), complete digestion was noted in six days while the average diameter of CXL corneas decreased by only 12% and the anterior curvature remained visible. In this study, corneas were obtained in 29 hours of death [24]. In another study, three CXL keratoconic corneas, three normal human corneas and three non-treated keratoconic corneas were evaluated. Keratoconic features were changed into normal features during CXL [24]. The samples were then subjected to enzymatic digestion by pepsin. Pepsin was used as it is a nonspecific endopeptidase that can break down both, collagen and proteoglycan core proteins. It is, therefore, more appropriate for assessing the effect of CXL than collagenase [24]. Normally, 90% of the cornea is formed by a stromal layer [25]. This regular thick connective tissue is formed by bundles of collagen, arranged in lamellae. In this study we have seen that CXL procedure increases the digestion time when corneas are treated with collagenase, pepsin and trypsin enzymes. This result explains the biomechanical effect of CXL treatment [19,26-28]. The stroma of the cornea forms about 90% of the thickness of the cornea. This transparent regular connective tissue is formed by bundles of collagen. Therefore, collagenase enzyme has more prominent effects on cornea than the other enzymes that we also had experienced in this study. Regular arrangement of the lamellae is another important feature of the histological appearance of stroma. In the collagenase treated corneas we have seen loss of this regularity and also seen spaces between lamellae as the result of digestion. Excluding the time period between the enzymes and control/ CXL corneas, graphics show that the digestion procedure was parallel in all groups. This means that diameter, curvature, transparency and digestion progresses in the same manner in all enzymes and control/CXL groups. The most apparent difference was the time period of digestion between these groups.

Our results should be interpreted in the light of its potential limitations. The major limitation of the current study is the sample size. The CXL group was treated with the RBF/ UVA and the control group was only treated with RBF. Another limitation is that different types of RBF solutions were not applied and compared. The main strength of our study is that the corneas were obtained from fresh-frozen human cadavers.

These findings also encourage the new treatment techniques of the corneal ulcers. In order to make reliable generalizations and statistics further studies with larger number of samples also evaluating ultrastructural changes of human corneas are needed.

Limitations of the study

The sample size in this study was very small to perform hypothesis tests. Findings should be considered as preliminary results and evaluated using clinical significance. More samples are required to assess statistical significance of the results.

Acknowledgment

The authors sincerely thank those who donated their bodies to science so that anatomical research could be performed. Results from such research can

© 2022 Acta Medica.

potentially increase mankind's overall knowledge that can then improve patient care. Therefore, these donors and their families deserve our highest gratitude.

Author contribution

Study conception and design: AF and ZYA; data collection: AF and ZYA; analysis and interpretation of results: AF, ÖD, and ZYA; draft manuscript preparation: AF, ÖD, ZYA, and HMH. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Ethics Committee of Faculty of Medicine, Hacettepe University (GO 13/275-34).

Funding

The authors declare that the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

[1] Wollensak G, Wilsch M, Spoerl E, et al. Collagen fiber diameter in the rabbit cornea after collagen crosslinking by riboflavin/UVA. Cornea 2004;23(5):503-507.

[2] Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. Curr Eye Res 2004;29(1):35-40.

[3] Wollensak G, Redl B. Gel electrophoretic analysis of corneal collagen after photodynamic cross-linking treatment. Cornea 2008;27(3):353-356.

[4] Arafat SN, Robert MC, Shukla AN, et al. UV cross-linking of donor corneas confers resistance to keratolysis. Cornea 2014;33(9):955-959.

[5] Smith VA, Hoh HB, Easty DL. Role of ocular matrix metalloproteinases in peripheral ulcerative keratitis. Br J Ophthalmol 1999;83(12):1376-1383.

[6] Sternberg SS, editor. Histology for Pathologists. Philadelphia, USA: Lippincott Williams&Wilkins, 1997.

[7] Zhu Y, Reinach PS, Zhu H, et al. High-intensity corneal collagen crosslinking with riboflavin and UVA in rat cornea. PLoS One 2017;12(6):e0179580.

[8] Fini ME, Girard MT, Matsubara M. Collagenolytic/gelatinolytic enzymes in corneal wound healing. Acta Ophthalmol Suppl 1992;202:26-33.

[9] Riley GP, Harrall RL, Watson PG, et al. Collagenase (MMP-1) and TIMP-1 in destructive corneal disease associated with rheumatoid arthritis. Eye (Lond) 1995;9(Pt 6):703-718.

[10] Schilde T, Kohlhaas M, Spoerl E, et al. Enzymatic evidence of the depth dependence of stiffening on riboflavin/UVA treated corneas. Ophthalmologe 2008;105(2):165-169.

[11] Kanellopoulos AJ, Loukas YL, Asimellis G. Cross-Linking Biomechanical Effect in Human Corneas by Same Energy, Different UV-A Fluence: An Enzymatic Digestion Comparative Evaluation. Cornea 2016;35(4):557-561.

[12] Kohlhaas M. Complications and postoperative therapeutic strategies in cross-linking. Ophthalmologe 2017;114(8):693-696.

[13] O'Brart NAL, O'Brart DPS, Aldahlawi NH, et al. An Investigation of the Effects of Riboflavin Concentration on the Efficacy of Corneal Cross-Linking Using an Enzymatic Resistance Model in Porcine Corneas. Invest Ophthalmol Vis Sci 2018;59(2):1058-1065.

[14] Knyazer B, Krakauer Y, Baumfeld Y, et al. Accelerated Corneal Cross-Linking With Photoactivated Chromophore for Moderate Therapy-Resistant Infectious Keratitis. Cornea 2018;37(4):528-531.

[15] Laggner M, Pollreisz A, Schmidinger G, et al. Correlation Between Multimodal Microscopy, Tissue Morphology, and Enzymatic Resistance in Riboflavin-UVA Cross-Linked Human Corneas. Invest Ophthalmol Vis Sci 2015;56(6):3584-3592.

[16] Brekelmans J, Veugen J, Rieff K, et al. Enzymatic Digestion of Porcine Corneas Cross-linked by Hypo- and Hyperosmolar Formulations of Riboflavin/ultraviolet A or WST11/Near-Infrared Light. Transl Vis Sci Technol 2020;9(10):4.

[17] Tan HY, Chang YL, Lo W, et al. Characterizing the morphologic changes in collagen crosslinked-treated corneas by Fourier transform-second harmonic generation imaging. J Cataract Refract Surg 2013;39(5):779-788.

[18] McCall AS, Kraft S, Edelhauser HF, et al. Mechanisms of corneal tissue cross-linking in response to treatment with topical riboflavin and long-wavelength ultraviolet radiation (UVA). Invest. Ophthalmol Vis Sci 2010;51(1):129-138.

[19] Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. Exp Eye Res 1998;66(1):97-103.

[20] Wollensak G, Sporl E, Reber F, et al. Corneal endothelial cytotoxicity of riboflavin/UVA treatment in vitro. Ophthalmic Res 2003;35(6):324-328.

[21] Wollensak G, Sporl E, Mazzotta C, et al. Interlamellar cohesion after corneal crosslinking using riboflavin and ultraviolet A light. Br J Ophthalmol 2011;95(6):876-880.

© 2022 Acta Medica.

[22] Kanellopoulos AJ, Asimellis G, Salvador-Culla B, et al. High-irradiance CXL combined with myopic LASIK: flap and residual stroma biomechanical properties studied ex-vivo. Br J Ophthalmol 2015;99(6):870-874.

[23] Alageel SA, Arafat SN, Salvador-Culla B, et al. Corneal Cross-Linking With Verteporfin and Nonthermal Laser Therapy. Cornea 2018;37(3):362-368.

[24] Hayes S, Kamma-Lorger CS, Boote C, et al. The effect of riboflavin/UVA collagen cross-linking therapy on the structure and hydrodynamic behaviour of the ungulate and rabbit corneal stroma. PLoS One 2013;8(1):e52860.

[25] Michael H. Ross LJR, Gordon I. Kaye, editors. Histology, A Text and Atlas. Maryland, USA: Wiliams&Wilkins, 1995.

[26] Akhtar S, Almubrad T, Paladini I, et al. Keratoconus corneal architecture after riboflavin/ultraviolet A cross-linking: ultrastructural studies. Mol Vis 2013;19:1526-1537.

[27] Carlson EC, Waring GO, 3rd. Ultrastructural analyses of enzyme-treated microfibrils in rabbit corneal stroma. Invest Ophthalmol Vis Sci 1988;29(4):578-585.

[28] Meek KM, Tuft SJ, Huang Y, et al. Changes in collagen orientation and distribution in keratoconus corneas. Invest Ophthalmol Vis Sci 2005;46(6):1948-1956.

© 2022 Acta Medica.