AMJ
ISSN: 0005-2523

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

Central Cornea Thickness and Keratocyte Cell Density After Transglutaminase-Induced Corneal Collagen Crosslinking

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Keywords:
Corneal Collagen Crosslinking, Transglutaminase, Riboflavin, Central Cornea Thickness, Keratocyte Cell Density

ABSTRACT

Corneal collagen crosslinking is a procedure for making bonds that connect polymer chains. Corneal collagen crosslinking aims to slow or stop the progression of keratoconus by using photooxidative therapy to increase stromal rigidity. This research is aimed to evaluate the effect of transglutaminase-induced corneal collagen crosslinking (CXL) on central corneal thickness and keratocyte cell density in vivo. Twenty-eight white New Zealand rabbits were divided into four groups: the transglutaminase-induced CXL group, the epithelial-off CXL group, the transepithelial CXL group, and the control group. The ocular surface was treated with a 1 U/mL microbial transglutaminase solution, and both the epithelial-off and transepithelial groups were exposed to clinical ultraviolet A-riboflavin (UVA/RF). The efficacy of each group was evaluated on the 14th day after the procedures. Central corneal thickness and keratocyte cell density were evaluated with histopathology examination. Transglutaminase-induced CXL group exhibited the highest mean central corneal thickness (341.10 ± 58.50) in comparison to the UVA/RF epithelial-off group (289.42 ± 38.19), the UVA/RF transepithelial group (319.15 ± 16.81) and control group (318.40 ± 63.97). Still, there was no significant difference, with a *p-value* of 0.279. Transglutaminase-induced CXL group had the highest mean of keratocyte cell density (43.26 ± 10.65) compared to UVA/RF epithelial-off (29.99 ± 4.79), UVA/RF transepithelial group (42.03 ± 6.55), and control group (34.36 ± 6.76). There was a significant difference between the group, with a *p-value* of 0.008. It implied that the effect of transglutaminase-induced CXL could be comparable to UVA/RF CXL in altering central cornea thickness and keratocyte cell density.

1. Introduction

Corneal collagen crosslinking (CXL) is one of the therapeutic methods in corneal ectasia, which aims to slow or stop the progression of keratoconus by using photooxidative therapy to increase stromal rigidity. In this procedure, a bond occurs that connects the polymer chains. This bond can be covalent or ionic [1], [2].

Currently, riboflavin and ultraviolet-A (UVA) are used in conventional CXL. Corneal collagen crosslinking with riboflavin is effective in strengthening the corneal tissue. However, the CXL method using riboflavin and UVA is considered too often to cause discomfort to patients due to UVA rays. [3].

Currently, there is an enzymatic material that can be used as an option for performing CXL. Enzymatic products, in the form of transglutaminase, are considered to reduce discomfort due to UVA irradiation, and can inhibit the death of keratocytes. It is envisaged that using accessible enzymatic products as a treatment technique will be easier and reduce the cost of CXL therapy [4], [5].

The cornea is a transparent and avascular tissue, nutrients and oxygenation of the cornea are obtained by diffusion through the aqueous humor, tear film, and limbal blood vessels [6] Corneal ectasia is a condition where the cornea's thickness decreases from normal values. One of the anticipated modifications following CXL's provision is this. The chromophore is absorbed by ultraviolet-A, which also triggers the lysyl oxidase pathway and results in crosslinking [7]. Corneal collagen crosslinking causes mechanical properties such as collagen fibers of the cornea to increase. Improved biomechanics can increase the cornea's rigidity, thereby increasing its thickness [8].

In several studies, corneal collagen crosslinking using ultraviolet A/riboflavin (UVA/RF) significantly damaged keratocytes. The formation of singlet oxygen, a bio-toxic gas that also causes collagen protein crosslinking, contributes to UVA-induced cell damage [9]. Apoptotic keratocytes can be replaced by precursor cells that can be activated under certain conditions, forming new cells and restoring the cornea's transparency [10].

In this study, New Zealand rabbits were divided into four groups, namely the transglutaminase-induced CXL group, Ultraviolet A/riboflavin (UVA/RF) epithelial-off group, Ultraviolet A/riboflavin (UVA/RF) transepithelial group, and control group. This study aims to evaluate the effect of transglutaminase-induced CXL on central corneal thickness and keratocyte cell density in vivo.

2. Materials and Methods

2.1 Subject and measures

Twenty-eight normal New Zealand white rabbits were enrolled, and the right eye was treated. All rabbits were male, between 3 and 4 months of age, and weighed between 3 and 4 kg. All rabbits were provided by the Faculty of Veterinary Medicine, Universitas Airlangga. This study was approved by the Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga. The experiments were performed at Veterinary Hospital Universitas Airlangga.

2.2 Crosslinking procedure

Anesthesia was induced via intramuscular injection of 0.1 mL/kgBW Xylazine 2% (Xyla, Interchemie, Netherland) and 6-10 mg/kgBW Ketamine (Ket-A-100®, Agrovet Market s.a. Lima, Peru). The right eyes were treated, and the contralateral eyes served as untreated controls kept closed during the procedure. The corneal epithelium of the first group was removed using an 8 mm MacRae Photorefractive Keratectomy (PRK) well and alcohol 20% for 40 seconds, then the loose epithelium was removed with a hockey knife. After the epithelial-off procedure, Transglutaminase solution 1U/mL (Zedira, Germany) was dripped onto the cornea every 2 minutes for 30 minutes, in total 16 times. The cornea epithelial of the transepithelial group was not removed, then the riboflavin transepithelial (Peschke TE, Peschke Meditrade GmbH, Switzerland) was dripped onto the cornea every 2 minutes for 30 minutes. Subsequently, UVA exposure

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

(365 ± 5 nm, 3 mW/cm2, 8-mm diameter light spot) was performed using a UVA lamp CCL-Vario System (Peschke Trade GmbH, Switzerland) while continuous drip the riboflavin onto the cornea every 2 minutes in 30 minutes. The cornea epithelial of the epithelial-off group was removed with the same procedure, then the riboflavin epithelial-off (Ribolink, Aurolab, India) was dripped two times onto the cornea. Subsequently, UVA exposure was performed using the same technique while continued to drip the riboflavin onto the cornea every 5 minutes for 30 minutes [11], [12]. Immediately following the procedure, an eye ointment containing chloramphenicol (Cendo, Indonesia) was used. The ointment was administered every eight hours for two weeks.

2.3 Central corneal thickness and keratocyte cell density evaluation procedure

Fourteen days after treatment, enucleation was performed, the corneal button was excised for corneal staining using hematoxylin eosin. Corneal thickness data (µm) is the average thickness in five fields of view at 100x magnification. The data of keratocyte cell density (µm2) is the mean value of keratocyte cell density in five different fields of view at 400x magnification. The mean keratocyte cell density is the average number of keratocytes in the corneal stroma area of 20,000 m2. All of these examinations used an ordinary Nikon Eclipse E-i light microscope equipped with a 300-megapixel DS Fi2 digital camera and a calibrated Nikon Image System image processing software.

2.4 Statistical analysis

Comparative data between were tested using the one-way ANOVA and will be continued with a post-hoc test if the data is normally distributed. If the data is not normally distributed, it will be analyzed using the Kruskal Wallis test and continued by using the Mann-Whitney test. The result is considered significant if the *p-value* is < 0.05. All statistical data were processed using SPSS 26.0 software (SPSS, Chicago. IL, USA).

3. Result

3.1 Comparison of central corneal thickness

Our study demonstrated that the transglutaminase-induced CXL group had the highest value of central corneal thickness, 459.12 µm. The lowest value was from UVA/RF epithelial-off group with 245.61 µm. Compared to the UVA/RF epithelial-off CXL group and the UVA/RF transepithelial CXL group, the central corneal thickness of the transglutaminase-induced CXL group displayed the highest mean score (341.10±58.50) (Table 1). The one-way ANOVA test analysis revealed no significant differences between any groups (*p-value* 0.279).

3.2 Comparison of keratocyte cell density

The research results showed that the keratocyte cell density was lowest in the UVA/RF epithelial-off CXL group with 23.39. The highest cell density was in the transglutaminase-induced CXL group, with 58.47. The mean keratocyte cell density in the transglutaminase-induced CXL group was the highest among the four groups (43.26±10.65) (Table 2). Analysis using a one-way ANOVA test revealed there were significant differences between any of the groups with *a p-value* of 0.008. A post-hoc test was performed, and there were significant differences between the UVA/RF epithelial-off CXL group and the UVA/RF transepithelial CXL group (*p-value* 0.003) and transglutaminase-induced CXL (*p-value* 0.006).

4. Discussion

This study performed a histological examination with hematoxylin-eosin staining to measure the central corneal thickness. With a value of 459.12 m, the analysis revealed that the transglutaminase-induced CXL

group had the thickest central cornea. With 245.61 m, the UVA/RF epithelial-off CXL group recorded the lowest value. According to statistical analysis, despite changes in central corneal thickness, particularly in UVA/RF epithelial-off CXL, there was no significant difference with a *p-value* of 0.279.

Research conducted by Wu et al. had similar results to this study, where there was no significant difference between the central corneal thickness of rabbits in the transglutaminase-induced CXL, UVA/RF CXL group and the control group. The lack of considerable central corneal thickening after two weeks may be related to epithelial remodeling and stromal oedema that occurred several days after treatment; as a result, the corneal thickness would require longer time [11], [13].

In a study conducted by Arance-Gil et al. stated that central corneal thickness decreased in the first few months after UVA/RF epithelial-off CXL was performed, then increased in the next twelve months. In the transepithelial method, the thickness of the central cornea tends to be more stable [13]. The decrease in central corneal thickness after CXL with the epithelial-off method can be caused by several factors, including compaction of collagen fibers, dehydrated corneal stroma, apoptosis of keratocytes caused by UVA rays. This can explain why in this study, CXL using UVA/RF epithelial-off had the lowest central corneal thickness, because in this method the corneal epithelial debridement procedure was carried out as well as UVA irradiation. Factors that trigger an increase in central corneal thickness can be caused by the repopulation of keratocytes, where this process takes a longer time, which is about six months [14].

Transglutaminase is an enzyme that catalyzes the formation of isopeptide bonds between proteins. This can occur through crosslinking of the protein due to the binding of -glutamyl lysine or due to the union of the primary amino acid with residues of the peptide and glutamine bonds [15]. Recent studies have shown that mRNA, fibronectin, and transglutaminase were found to be more abundant in human corneal keratocytes treated with UVA and riboflavin. Induction by transglutaminase is proposed as a new mechanism for crosslinking. The treatment of CXL transglutaminase did not use UVA irradiation, this could be the cause of the central corneal thickness in the transglutaminase group having the highest average [12], [16].

In 2016 Antonios et al. compared several devices to measure central corneal thickness in patients with keratoconus after CXL. The results obtained in this study were that the central corneal thickness began to show a minimal increase in the second week, and a significant increase was only obtained three months after the procedure. The thickness of the central cornea in the early postoperative week did not experience a significant increase which could be due to stromal opacity, which affected how some equipment read the results [17].

The statistical analysis showed a significant difference in the keratocyte cell density between the treatment and control groups, with a p-value of 0.008. After the post-hoc test, it was found that there were significant differences between the UVA/RF CXL epithelial off group with UVA/RF transepithelial (*p-value* 0.003) and transglutaminase-induced CXL (*p-value* 0.006).

The UVA/RF CXL treatment group using the epithelial-off approach had the lowest mean value of keratocyte cell density, according to this study. This might be as a result of corneal debridement and UVA radiation given to the epithelial-off group. This is described in the study by Mencucci et al. (2010), where the epithelial debridement and UVA light resulted in a reduction of keratocyte cell density [10], [18].

The research of Wu et al. found that the UVA/RF CXL group found a decrease in keratocyte density. In contrast, the transglutaminase-induced CXL group did not find a decrease in the number of keratocyte cell

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

density, or cell apoptosis, although the difference was not statistically significant. The factor that causes the stability of keratocyte cell density in transglutaminase-induced CXL is the method does not use UVA, which can induce photochemical reactions that can reduce the keratocyte cell density [19]. UVA can induce reactive oxygen species (ROS), which not only causes crosslinking of collagen but can also cause necrosis and apoptosis of cells in the eyeball, one of which is the keratocytes in the corneal stroma. Irradiation using UVA has a standard dose of 3 mW/cm2 to induce crosslinking, then the dose is six times greater than the dose of UVA light, which can cause a cytotoxic effect of 0.5mW/cm2 [20], [21].

A study by Mencucci et al. also said the same regarding UVA/RF CXL, where UVA can stimulate apoptosis of keratocytes but also cause repopulation. Apoptosis of keratocytes occurs mainly in the first 24 hours after the procedure. The results of the analysis on the cornea six months after UVA/RF CXL showed that apoptotic cells were still present compared to the cornea in the control group. A study by Wollensak et al. explained that riboflavin is not a cytotoxic agent for the cornea, but riboflavin is a photosensitizing agent that can help UVA rays to be better absorbed by the cornea [9], [10].

The limitation of current study

The study's disadvantages include the small sample size and brief study period, which make it challenging to detect changes in central corneal thickness and keratocyte cell density at two weeks post-op. The keratocyte density examination also employs hematoxylin-eosin staining and manual computations, which limits its ability to assess cell apoptosis to keratocyte density alone.

5. Conclusions

This study implies that the effect of transglutaminase-induced CXL could be comparable to UVA/RF CXL in terms of altering central cornea thickness and keratocyte cell density.

6. Acknowledgements

Surabaya Eye Clinic for lending CCL Vario System; Dr. Mahmudah, Ir., MKes (Faculty of Public Health – Universitas Airlangga) for statistical and methodology advice, Dr. Djoko Legowo, drh., M.Kes (Faculty of Veterinary Medicine – Uvinersitas Airlangga) for histopathological examination advice

7. Funding Information

This research received grant of Faculty Excellence in Reasearch Awards from Universitas Airlangga (No. 1331/UN3.1.1/HK/2022)

8. Ethical Standard

Ethical approval was obtained from Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga (No. 2.KEH.071.07.2022). All Procedures were performed with ethical standards

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Table 1: Comparison of central corneal thickness

	Mean	Std. Deviation	Minimum	Maximum	*p-value*
Transglutaminase	341.10	58.50	291.84	459.12	0.279
Riboflavin/UVA *epithelial-off*	289.42	38.19	245.61	348.83	
Riboflavin/UVA *transepithelial*	319.15	16.81	291.29	341.61	
Kontrol negatif	318.40	63.97	187.12	388.74	

Table 2: Comparison of keratocyte cell density

	Mean	Std. Deviation	Minimum	Maximum	*p-value*
Transglutaminase	43.26	10.65	32.37	58.47	0.008
Riboflavin/UVA *epithelial-off*	29.99	4.79	23.39	37.56	
Riboflavin/UVA *transepithelial*	42.03	6.55	31.88	50.47	
Kontrol negatif	34.36	6.76	27.20	46.42	

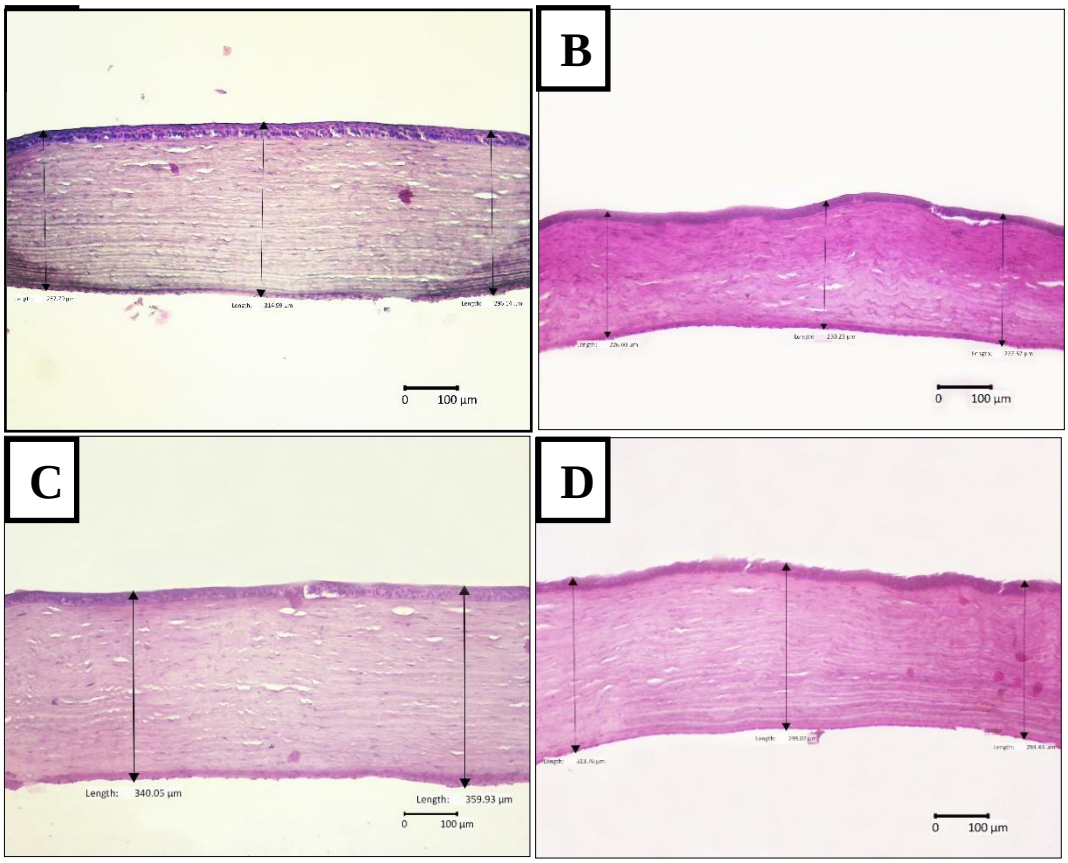

Figure 1. Central corneal thickness comparison between groups (A) transglutaminase-induced CXL group (B) UVA/RF epithelial-off CXL group (C) UVA/RF transepithelial CXL group (D) control group (HE staining; 40x objective lens; bar = 50µm; Nikon Eclipse E-i microscope; 300 megapixel DS Fi2 camera)

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ISSN: 0005-2523
ISSN: 0005-2523
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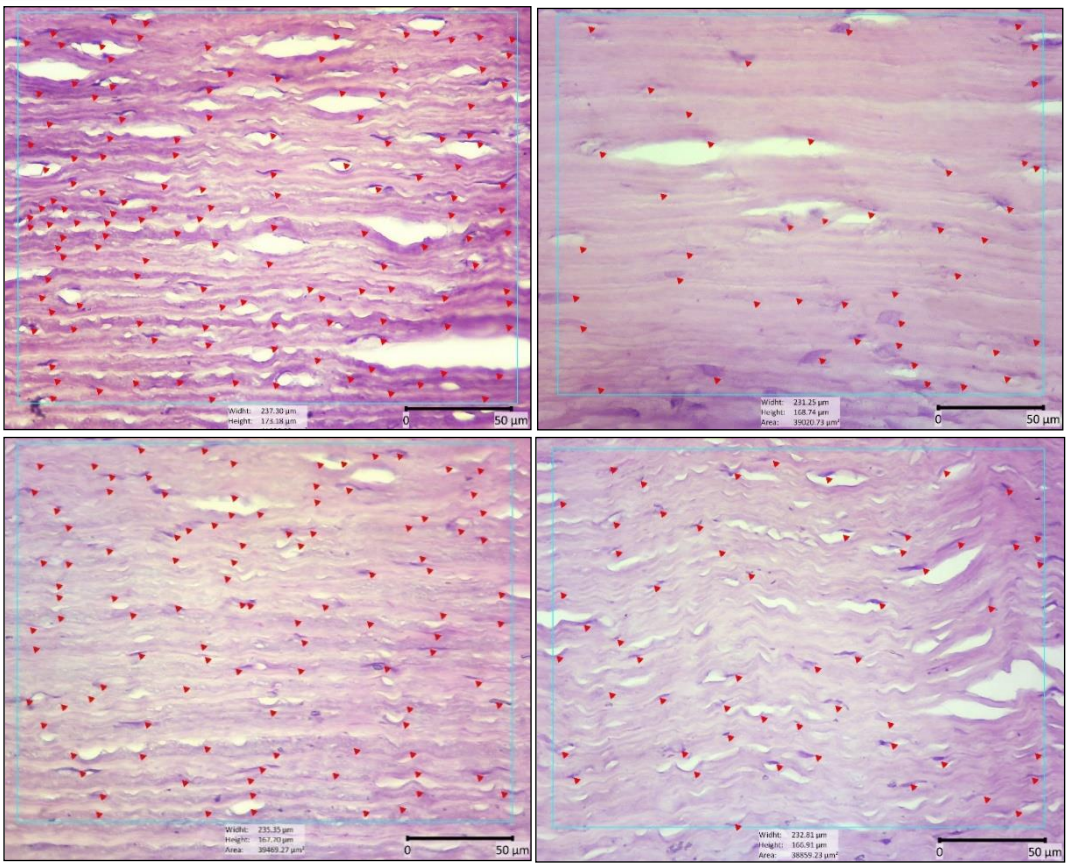

Figure 2. Comparison of keratocyte cell density between groups (A) transglutaminase-induced CXL group (B) UVA/RF epithelial-off CXL group (C) UVA/RF transepithelial CXL group (D) control group