Int Ophthalmol (2019) 39:1329–1335
https://doi.org/10.1007/s10792-018-0951-x

ORIGINAL PAPER

Corneal stromal depth of the demarcation line in 'accelerated corneal cross-linking' with different concentrations of riboflavin solutions

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Received: 12 June 2017 / Accepted: 8 June 2018 / Published online: 14 June 2018

Abstract

Purpose The aim of this study is to compare the effect of different riboflavin solutions (hypotonic and isotonic) used during accelerated corneal cross-linking (CXL) on the mean depth of the demarcation line (DDL) formed in corneal stroma.

Methods This prospective, cross-sectional study included 38 eyes of 26 patients. All patients underwent accelerated CXL due to progressive keratoconus. When the corneal epithelium was removed, 17 eyes of 12 patients with corneal thickness < 400 μm were categorized as Group 1, and 21 eyes of 14 patients with corneal thickness > 400 μm as Group 2. Hypotonic riboflavin was applied to Group 1 patients, and isotonic riboflavin to Group 2 patients. Anterior segment optical coherence tomography was performed on all patients by two independent observers at the end of the first and third months.

Result Group 1 included 5 male and 7 female patients with an average age of 25.1 ± 8.0 years, whereas Group 2 included 7 male and 7 female patients with an average age of 31.8 ± 10.12 years. At the end of the first month, the mean DDL in Group 1 and Group 2 was 180.32 ± 10.26 and 287.21 ± 15.01 μm, respectively. This difference was statistically significant ($p < 0.05$).

Conclusion Application of different riboflavin solutions was observed to have an effect on measured corneal thickness after saturation and the depth of the demarcation line. The use of hypotonic riboflavin results in swelling of the cornea and more superficial localization of the stromal demarcation line after CXL.

Keywords Keratoconus · Accelerated corneal cross-linking · Riboflavin · Demarcation line

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Introduction

Keratoconus is the most common primary ectasia and is characterized by corneal steepening, visual distortion, apical corneal thinning, and central corneal scarring. Over the last decade, CXL has become a conventional treatment method for progressive

keratoconus. The primary aim of CXL is to stop the progression of corneal ectasia. To strengthen corneal tissue, riboflavin is combined with ultraviolet-A irradiation (UV-A). Riboflavin functions as a photosensitizer in the photopolymerization process and when combined with UV-A irradiation, increases the formation of intrafibrillar and interfibrillar carbonyl-based collagen covalent bonds through a molecular process that has still not been completely elucidated [1]. Recent studies have demonstrated that CXL treatment improves corneal rigidity [2, 3] and increases the corneal resistance to enzymatic digestion [4].

The demarcation line is one of the best indicators of the effectiveness of the CXL. In this study, we investigated the comparative impact of the utilization of hypotonic and isotonic riboflavin in accelerated corneal cross-linking (CXL) on the mean depth of the demarcation line (DDL).

Patients and methods

This prospective, single-center, comparative study was conducted on patients who underwent CXL from December 2017 through March 2017 of Ophthalmology, Ankara Numune Research and Training Hospital, Turkey. This study was approved by the local Ethics Committee of Ankara Numune Research and Training Hospital and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

The study included 38 eyes of 26 patients, all of whom underwent accelerated CXL due to progressive keratoconus. Progression was defined as an increase of Kmax over 1D (diopter), an increase of $> 2\%$ thinning on the thinnest point of the cornea and > 0.5D increase on manifested spherical refractive value on Scheimpflug camera (Pentacam HR, Oculus Optik geräte GmbH, Wetzlar Germany) evaluation. When the corneal epithelium was removed, 17 eyes of 12 patients with corneal thickness < 400 μm were categorized as Group 1, and 21 eyes of 14 patients with corneal thickness > 400 μm as Group 2. Hypotonic riboflavin was applied to Group 1 patients and isotonic riboflavin to Group 2 patients. Anterior segment optical coherence tomography (AS-OCT) was performed on all patients by two independent observers at the end of the first and third months.

Patients with a history of previous anterior segment surgery, ocular surface problems, herpetic keratitis, active ophthalmic infection or any central or paracentral corneal scar were not included in the study (Figs. 1, 2).

Corneal thickness in Group 1 eyes was measured to be between > 330 and < 400 μm, whereas in Group 2 eyes, corneal thickness was above 400 μm during the epithelium-off accelerated CXL operation.

Treatment modalities, medical history review, uncorrected visual acuity (UCVA), corrected visual acuity (CDVA), slit-lamp and fundus examination findings, and central corneal thickness at the thinnest point (t-CCT) were recorded preoperatively, at the end of the first and third months postoperatively.

AS-OCT (Casia swept-source OCT-1000, Tomey, Nagoya, Japan) was applied postoperatively to all patients at the end of the first and third months under similar light conditions. The depth of the stromal demarcation line was measured using the caliper tool from the manufacturer which revealed that it was identified within an enhanced image of the cornea on the horizontal meridian. The DDL on AS-OCT from the two examiners were identical in all eyes examined at the end of the first and third months.

Surgical procedure and postoperative treatment

Before the CXL operation, proparacaine hydrochloride (0.5%) (Alcaine; Alcon Laboratories, Puurs, Belgium) was applied. The operation started with central 8.0-mm corneal epithelium debridement with a crescent knife (Beaver–Visitec International Inc., Waltham, MA) with the benefit of 20% alcohol applied for 30 s with trephine. Afterward, 5% NaCL solution was used for thorough cleaning. Hypoosmolar 0.1% riboflavin (without dextran, MedioCROSS® H, Avedro Inc., USA) treatment was started immediately after epithelium removal with applications at 2-min intervals until the minimal corneal thickness reached 400 mm. The corneal pachymetry was measured using an ultrasound (US) probe (SP-2000, Tomey, Inc.) preoperatively, after epithelial removal, and at every 10 min thereafter. At each time point, 10 measurements were performed in the thinnest area and the lowest pachymetry value of each measurement was recorded. Slit-lamp biomicroscopy (using cobalt blue) was used to ensure the successful penetration of riboflavin through the cornea

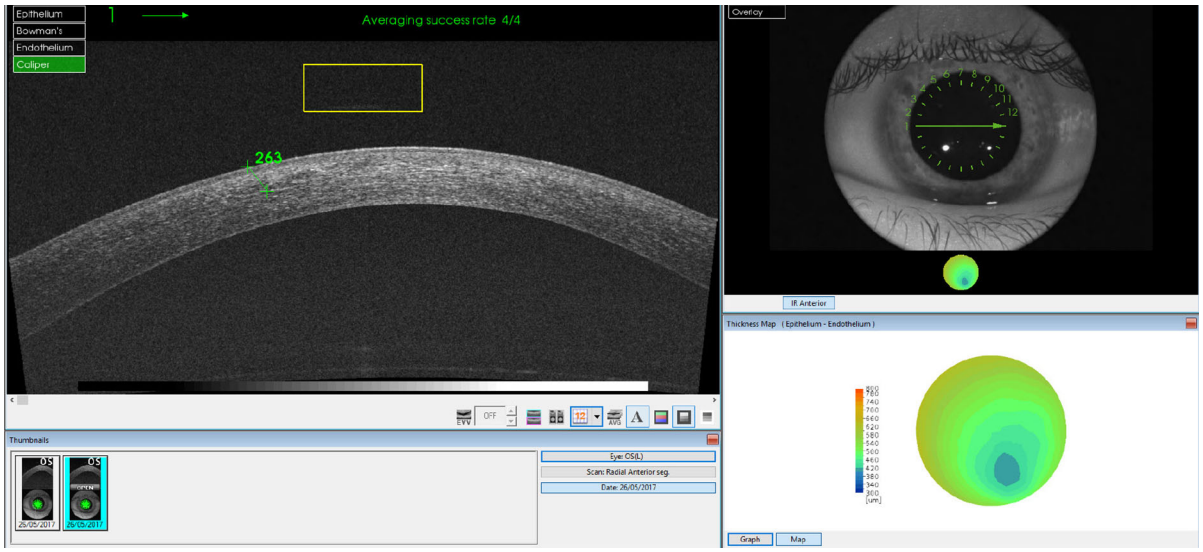

Fig. 1 Corneal stromal demarcation line image with anterior segment optical coherence tomography 1 month after accelerated corneal collagen cross-linking using isotonic solution (10 min at 9 mW/cm^2, total surface dose of 5.4 J/cm^2)

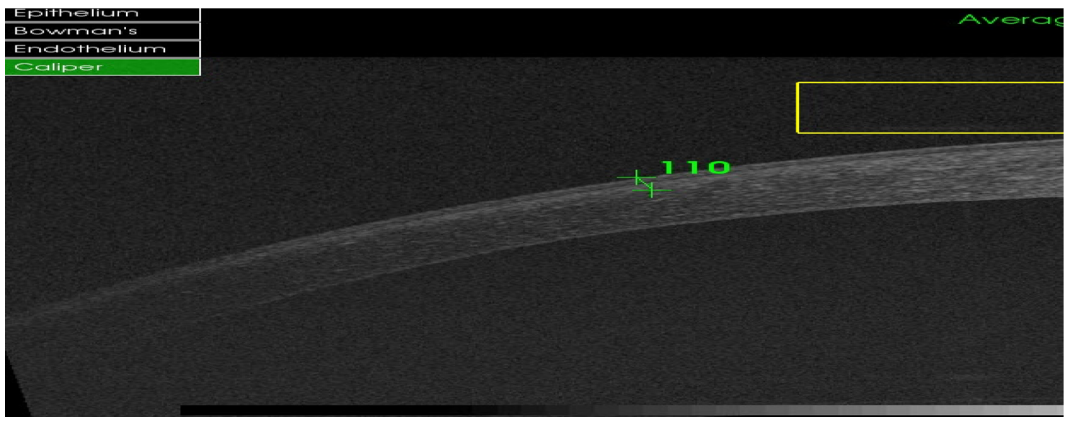

Fig. 2 Corneal stromal demarcation line image with anterior segment optical coherence tomography 1 month after accelerated corneal collagen cross-linking using hypotonic solution (10 min at 9 mW/cm^2, total surface dose of 5.4 J/cm^2)

by visualization of riboflavin in the anterior chamber. Riboflavin continued to be applied at 2-min intervals as well during the course of a 10-min exposure to 9 mW/cm^2 UV-A. Finally, a therapeutic contact lens (Air Optics; Alcon, Inc.) was fitted. During the treatment period, both eyes were treated with Diclofenac (Acular LS®) 4 × 1 and Netilmicin (Netira®) 4 × 1, loteprednol (Lotemax®, Bausch and Lomb Inc.) and artificial tears 6 × 1 as standard. Until a complete re-epithelization was observed, all patients underwent regular follow-up examinations on a daily basis. Antibiotic therapy was used for 1 week, a steroid tapering schedule was applied for 3 months, and artificial tears were continued for 6 months.

The data were analyzed using SPSS 20.0 for Mac software (SPSS, Inc, Chicago, IL, USA). The independent samples *t* test was applied in the comparisons of t-CCT and DDL. A value of $p < 0.05$ was considered statistically significant.

Results

Group 1 included 17 eyes of 12 patients, comprising 5 males, and 7 females with a mean age of

25.1 ± 8.02 years. Group 2 included 21 eyes of 14 patients, comprising 7 males and 7 females with a mean age of 31.8 ± 10.12 years. All patients underwent epithelium-off accelerated CXL. The clinical properties of the patients are shown in Table 1.

The pre-CXL Flattest K and Steepest K values in Group 2 were significantly higher than the post-CXL values ($p < 0.05$).

The t-CCT values in both groups before and after CXL are shown in Table 2. The measurements in both groups in terms of t-CCT values were similar before and after the treatment, at the end of the first and third months.

The experimental results as indicated in Table 2 reveal that the utilization of isotonic or hypotonic riboflavin resulted in no statistically significant differences in t-CCT. The mean depth values of the demarcation line at the end of the first month in both groups are shown in Table 3.

The difference in demarcation line depth between groups was statistically significant ($p < 0.05$, independent samples t test). This finding indicated that the effect of UV-A on corneal stroma was more superficial with the use of hypotonic solution. As a consequence, the biomechanical effect of cross-linking was on the anterior corneal stroma. At the end of the third month after CXL, the demarcation line appeared in 4 of 17 eyes in Group 1 and Group 2 of 21 eyes in Group 2. The number of sample eyes at the end of the third month was insufficient for any statistical conclusion.

The results of this study revealed a positive correlation between DDL and the minimum corneal thickness after epithelial removal (Spearman rank correlation coefficient $r = 0.42$, $p = 0.03$).

Table 1 Pre-CXL and post-CXL clinical properties of patients

	Group 1	Group 2
Pre-CXL astigmatism (D)	3.93 ± 1.34	4.82 ± 1.47
Pre-CXL flattest K (D)	47.23 ± 0.45*	50.41 ± 1.45
Steepest K (D)	50.45 ± 11.78*	59.12 ± 14.12
Pre-CXL UCVA (logMAR)	1,01 ± 0.88	1.12 ± 0.13
Pre-CXL BCVA (logMAR)	0.71 ± 1.40*	0.81 ± 1.1

*$p < 0.05$

CXL cross-linking; *K* keratometry; *D* diopter; *UCVA* uncorrected visual acuity; *BCVA* best corrected visual acuity

The therapeutic contact lenses were well tolerated by all the patients. No problems of epithelial healing were observed after the treatment. No complications were noted, such as infection, contact lens damage, or corneal infiltration.

Discussion

Corneal ectasia is the irregular protrusion of the cornea as a result of changes in stromal collagen matrix. The primary forms of corneal ectasia are keratoconus, pellucid marginal degeneration and keratoglobus, and the secondary form is ectasia after refractive surgery [1]. Depending on the activity and stage of keratoconus, eye glasses, contact lenses, intrastromal corneal ring segments, phakic intraocular lenses, photorefractive keratectomy, and corneal transplantation at the final stage are utilized in order to improve visual acuity [5].

In general, the progression in keratoconus is controlled by improving the biomechanical strength and stability of the cornea in CXL. The application of riboflavin and ultraviolet-A to reform new covalent bands is commonly performed by applying a relatively new approach named CXL. At different stages of keratoconus, the complications rates of CXL may vary between 1 and 10% [6].

The outcomes of accelerated CXL were examined using hypo-osmolar riboflavin solution on < 400 μm and isotonic riboflavin in > 400 μm thickness corneas. Using hypo-osmolar and isotonic dextran free riboflavin, 9mW/cm2 UV-A was applied and changes in corneal thickness and demarcation line were evaluated.

Endothelium decompensation is a significant complication of CXL. When the cornea is saturated with riboflavin to 400 μm thickness, UV-A radiance at the endothelial level is lower than at the cytotoxic level. This suggests that 400 μm thickness of the corneal stroma after the removal of the epithelium is a safe limit to protect the endothelium and intraocular structures from the adverse effects of UV-A irradiation. To protect patients with corneal thickness of < 400 μm against toxicity, many different methods have been proposed. Among these methods, there are some new techniques such as the use of hypo-osmolar riboflavin solution to inflate the cornea during the operation, transepithelial CXL without removal of the

Table 2 Change of t-CCT in CXL application

	Group 1	Group 2	*P*
Pre-CXL t-CCT	410.5 ± 11.6	480.3 ± 6.56	0.00
After removed epithelium t-CCT	378.5 ± 22.0	421.5 ± 11.7	0.04
End of the influx of the cornea with riboflavin	403.4 ± 5.7	461.5 ± 21.4	0.00
Post-CXL first month t-CCT	398.3 ± 9.0	469.1 ± 12.5	0.01
Post-CXL third month t-CCT	399.0 ± 11.2	468.9 ± 10.4	0.03

CXL cross-linking; *CCT* central corneal thickness

Table 3 DDL in the first month post-CXL

	Group 1	Group 2	*P*
DDL at the first month (μm)	180.32 ± 10.26	287.21 ± 15.01,	0.032

DDL depth of demarcation line

corneal epithelium, and customized epithelial debridement [7, 8]. Therefore, in this study, for corneal thickness of < 400 μm, the use of hypotonic riboflavin was preferred from these methods.

At the first stage of the CXL procedure, irrigation of the corneal stroma is used to increase the corneal thickness. A low colloid osmotic pressure can be used to swell the corneal stroma. Therefore, a hypo-osmolar solution is used in thin cornea, and the cornea is swollen with this hypo-osmolar solution after cornea de-epithelization [9]. In the current study, after de-epithelization, the cornea thickness increased from 378.5 ± 22.0 to 403.4 ± 5.7 μm using hypo-osmolar riboflavin solution. By increasing the thickness of the cornea sufficiently, it was aimed to avoid any complications.

The use of hypotonic riboflavin solution increased corneal pachymetry, although the final t-CCT remained unchanged. These results were seen to be compatible with the results reported in the study by Schmidingeret al in terms of CXL outcomes using hypotonic riboflavin solution [10]. In both cases, there were no statistically significant changes of t-CCT between pre-CXL and post-CXL. Similarly, no statistically significant differences were reported in the current study between the groups who underwent CXL with isotonic or hypotonic riboflavin solutions, in terms of the change in t-CCT level measurements from baseline preoperatively to post-CXL in the first and third months.

As has been reported in previous studies [11, 12], it was observed that the use of hypo-osmolar riboflavin solution does not change the efficiency of the treatment in respect of corneal parameters and visual performance. Although theoretically, as the hydrophilic capacity of the proteoglycans between the collagen fibers and the distance between the collagen fibrils increases in a swollen stroma, an increase in the distance between the collagen fibrils is expected as the result of swelling in stroma. This prevents the formation of covalent bands between collagen fibrils.

As the anterior stroma is more important in terms of biomechanical stability of the cornea, despite a significantly lower DDL depth after accelerated CXL, the cross-linking effect may also be strong enough to stop the progression of corneal ectasia, which should nevertheless be validated by future long-term clinical studies [13]. A higher UV intensity in the anterior cornea has a greater stiffening effect within the first 250 μm [14]. Oxygen is important for the photochemical polymerization reaction in CXL. Efficiency reduces in accelerated CXL because there is not enough time for oxygen diffusion [15].

DDL is a transition zone between the cross-linked stroma and untreated tissue. Most authors claim that the corneal stromal demarcation line can be used as a clinical sign to directly monitor the effective depth of the CXL treatment [2, 16]. It is generally assumed that the effect of DDL after CXL should be related to CXL [17–19]. However, there is no consensus that the corneal stromal DDL is an indicator of CXL efficacy. Thus, preferring deeper demarcation lines is not a reliable consideration in clinical approaches [20].

Previous studies have compared conventional CXL treatment showing DDL (30 min CXL performed in accordance with the Dresden protocol) and accelerated high-intensity 10-min CXL [21, 22]. After CXL using AS-OCT, the DDL was observed to have the highest visibility at the end of the first month of treatment. In a similar study, the UV-A light was used at 370 nm wavelength and 3mW/cm2 for the corneal cross-linking procedure, and the average depth was measured as 313 mm [23]. In the current study, the visibility of DDL after the first month was better compared to the third month, when the visibility was seen to have significantly decreased.

Kymionis et al. [16] reported that at the end of the first month, the DDL was 350 μm if standard CXL was applied, whereas it decreased to 288 μm in accelerated CXL. Tomita et al. [21] observed that at the end of the first month, the DDL was 350 μm in standard CXL, and 294 μm in accelerated CXL. Mazzotta et al. [24] measured the demarcation line depth pulse to be 215 μm in accelerated CXL, and in continued-accelerated CXL to be 160 μm. As an outcome, more singlet oxygen release for the cross-linking of collagen molecules has been observed which could be attributed to the additional oxygen re-diffusion during pauses. It was shown by Moramarco et al. [18] that the mean DDL value of 149 μm in a continued-accelerated CXL group and 213 μm in a pulse-accelerated CXL group indicated the importance of performing AS-OCT less than 1 month after CXL to obtain the most accurate value of DDL. In a study by Ozgur et al. [13], the DDL value 1 month after accelerated CXL was 205.64 ± 18.50 μm in thin keratoconic cornea. In the present study, the mean DDL was 180.32 ± 10.26 μm using hypotonic riboflavin and 287.21 ± 15.01 using isotonic riboflavin at the end of the first month in the accelerated CXL. In comparison with previous studies, the UV-A effect on the cornea was more superficial in the hypotonic group of the current study.

This retrospective interventional case series included patients with thin keratoconic corneas who received an accelerated CXL treatment at the Beyoglu Eye Training and Research Hospital, Istanbul, Turkey. All patients were informed before their participation in the study and provided written informed consent in accordance with the tenets of the Declaration of Helsinki. The study was approved by the ethics committee of the Beyoglu Eye Training and Research Hospital.

The findings of this study showed that corneal accelerated CXL treatment produces a superficial demarcation line in thin cornea and created no changes in t-CCT. The use of hypotonic riboflavin can be considered to have produced a similar effect by causing an increase in the corneal distance of the collagen fibers.

When it is taken into consideration that the anterior stroma is more important in terms of biomechanical stability of the cornea, the desired effect can be created using both isotonic and hypotonic riboflavin.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The study was conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent It was obtained from all individual participants included in the study.

References

1. Ziaei M, Barsam A, Shamie N et al (2015) Cornea clinical committee reshaping procedures for the surgical management of corneal ectasia. J Cataract Refract Surg 41:842–872
2. Seiler T, Hafezi F (2006) Corneal cross-linking-induced stromal demarcation line. Cornea 25:1057–1059
3. Dupps WJ Jr, Netto MV, Herekar S, Krueger RR (2007) Surface wave elastometry of the cornea in porcine and human donor eyes. J Cataract Refract Surg 23:66–75
4. Spoerl E, Wollensak G, Seiler T (2004) Increased resistance of crosslinked cornea against enzymatic digestion. Curr Eye Res 29:35–40
5. Park J, Gritz DC (2013) Evolution in the use of intrastromal corneal ring segments for corneal ectasia. Curr Opin Ophthalmol 24:296–301
6. Wollensak G, Spoerl E, Seiler T (2003) Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. Am J Ophthalmol 135:620–627
7. Wollensak G, Spoerl E, Reber F, Seiler T (2004) Keratocyte cytotoxicity of riboflavin/UVA treatment in vitro. Eye 18:718–722
8. Wollensak G, Spoerl E, Wilsch M, Seiler T (2003) Endothelial cell damage after riboflavin-ultraviolet: a treatment in the rabbit. J Cataract Refract Surg 29:1786–1790

9. Maurice DM, Giardini AA (1951) Swelling of the cornea in vivo after the destruction of its limiting layers. Br J Ophthalmol 35:791–797
10. Schmidinger G, Pachala M, Prager F (2013) Pachymetry changes during corneal crosslinking: effect of closed eyelids and hypotonic riboflavin solution. J Cataract Refract Surg 39:1179–1183
11. Hafezi F, Mrochen M, Iseli HP, Seiler T (2009) Collagen crosslinking with ultraviolet-A and hypoosmolar riboflavin solution in thin corneas. J Cataract Refract Surg 35:621–624
12. Raiskup F, Spoerl E (2011) Corneal cross-linking with hypo-osmolar riboflavin solution in thin keratoconic corneas. Am J Ophthalmol 152:28–32
13. Ozgurhan EB, Sezgin Akcay BI, Yildirim Y, Karatas G, Kurt T, Demirok A (2014) Evaluation of corneal stromal demarcation line after two different protocols of accelerated corneal collagen cross-linking procedures using anterior segment optical coherence tomography and confocal microscopy. J Ophthalmol 2014:981893
14. Raiskup F, Spoerl E (2013) Corneal crosslinking with riboflavin and ultraviolet A. I. Principles. Ocul Surf 11:65–74
15. Richoz O, Hammer A, Tabibian D, Gatzioufas Z, Hafezi F (2013) The biomechanical effect of corneal collagen cross-linking (CXL) with riboflavin and UV-A is oxygen dependent. Transl Vis Sci Technol 2:6–8
16. Kymionis GD, Tsoulnaras KI, Grentzelos MA et al (2014) Corneal stroma demarcation line after standard and high-intensity collagen crosslinking determined with anterior segment optical coherence tomography. J Cataract Refract Surg 40:736–740
17. Yam JC, Chan CW, Cheng AC (2012) Corneal collagen cross-linking demarcation line depth assessed by Visante OCT after CXL for keratoconus and corneal ectasia. J Refract Surg 28:475–481
18. Moramarco A, Iovieno A, Sartori A, Fontana L (2015) Corneal stromal demarcation line after accelerated crosslinking using continuous and pulsed light. J Cataract Refract Surg 41:2546–2551
19. Doors M, Tahzib NG, Eggink FA, Berendschot TT, Webers CA, Nuijts RM (2009) Use of anterior segment optical coherence tomography to study corneal changes after collagen cross-linking. Am J Ophthalmol 148:844–851
20. Gatzioufas Z, Balidis M, Kozeis N (2016) Is the corneal stromal demarcation line depth a true indicator of corneal collagen crosslinking efficacy. J Cataract Refract Surg 42:804
21. Tomita M, Mita M, Huseynova T (2014) Accelerated versus conventional corneal collagen crosslinking. J Cataract Refract Surg 40:1013–1020
22. Wernli J, Schumacher S, Spoerl E, Mrochen M (2013) The efficacy of corneal cross-linking shows a sudden decrease with very high intensity UV light and short treatment time. Invest Ophthalmol Vis Sci 54:1176–1180
23. Doors M, Tahzib NG, Eggink FA, Berendschot TTJM, Webers CAB, Nuijts RMMA (2009) Use of anterior segment optical coherence tomography to study corneal changes after collagen cross-linking. Am J Ophthalmol 148:844–851
24. Mazzotta C, Traversi C, Paradiso AL, Latronico ME, Rechichi M (2014) Pulsed light accelerated crosslinking versus continuous light accelerated crosslinking: one-year results. J Ophthalmol 2014:604731