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Original Article

CORNEAL COLLAGEN CROSSLINKING FOR THE TREATMENT OF MICROBIAL KERATITIS

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Abstract

Introduction: *Collagen cross-linking (CXL) is a new horizon in the treatment of corneal diseases. The corneal stroma is the thickest part of the cornea and is mainly composed of collagen fibrils, charged with stromal maintenance and wound healing. CXL by using UV rays and riboflavin could act as a photo mediator to inactivate pathogens in plasma, platelets, and red blood cells. Riboflavin also induces a change in properties of the collagen and has a stiffening effect on the corneal stroma, which stabilizes it and increases its resistance to enzymatic bacteria degradation avoiding the progression of corneal melting this study aimed to assess the efficacy and safety of corneal collagen cross-linking (CXL) in the management of infectious keratitis.* ***Patients and methods:*** *This study is prospective Interventional Non-comparative case series and included 16 eyes of 16 patients with clinical and lab (direct smear, culture, and sensitivity) evidence of microbial keratitis who attended the outpatient Cornea Unit, Ophthalmology department, Faculty of Medicine in Assiut University Hospital, Assiut. Data collected were detailed history and complete eye examination, including uncorrected visual acuity (UCVA), slit-lamp biomicroscopy, fundus examination, and intraocular pressure measurement for all patients.* ***Results:*** *Baseline visual acuity was in form of hand motion in 6 (37.5%) patients and bad perception of light (PL) in 5 (31.3%) patients. Follow-up visual acuity was in form of good PL IN 4 (25%) patients and 1m/60 in 3 (18.8%) patients. There was a significant improvement in the visual acuity during follow-up in comparison to baseline visual acuity (2.83 ± 0.70 vs. 1.93 ± 0.84; P< 0.001).* ***Conclusion:*** *CXL appears to be an effective procedure in treating non-resolving microbial keratitis with superficial stromal involvement. The most promising results published so far are for keratitis especially when the germs do not involve the posterior stroma and in cases of impending perforation.*

Keywords: *Keratoconus, Anterior corneal astigmatism, Posterior corneal astigmatism*

1. Introduction

Microbial keratitis is a leading cause of ocular morbidity and blindness around the world. It has been assessed that about 50% of the eyes have poor visual outcomes if the diagnosis and initiation of appropriate antimicrobial treatment are postponed [1]. Infectious corneal diseases are treated by topically antibiotics. The emergence of multidrug-resistant bacteria is a worry that could muddle the treatment. Some microbial keratitis resistant to the new antibiotics has been recently described [2]. Collagen

cross-linking using ultraviolet and riboflavin eye drops is a treatment that was developed to increase the biomechanical strength of the cornea thus blocking the progression of keratoconus [3]. The procedure is based on utilizing riboflavin photosensitizer which create reactive oxygen species when activated by UV-A at 365 or 370 nm. By way of photochemical reactions, these give rise to covalent bonds of cross-links in the corneal stroma [4]. Photoactivation of riboflavin induces oxidative stress. Since the 1960_s the idea of combining riboflavin with UV-A light has been explored methodically. The procedure was used to treat tobacco plants infected with tobacco mosaic virus [5]. Ultraviolet A light was utilized to inactivate microorganisms in drinking water and to disinfect blood products [6]. Riboflavin prompts a change in properties of the collagen and affects the corneal stroma, which stabilizes it and increases its stiffness and resistance to enzymatic bacterial degradation avoiding the progression of corneal melting [7]. In practice, the standard riboflavin/ultraviolet A crosslink protocol seems to produce only short-term improvement in pain, irritation, and discomfort and sometimes reducing the corneal thickness [8]. This study aimed to evaluate the efficacy and safety of corneal collagen cross-linking (CXL) in the treatment of resistant microbial keratitis.

2. Patients and Methods

This study is prospective Interventional Non-comparative case series and included 16 eyes of 16 patients with clinical and laboratory (direct smear, culture, and sensitivity) evidence of microbial keratitis who attended the outpatient Cornea Unit, Ophthalmology department, Faculty of Medicine in Assiut University Hospital, Assiut between January 2019- December 2020. Data collected were detailed history and complete eye examination, including uncorrected visual acuity (UCVA), slit-lamp biomicroscopy, fundus examination, and intraocular pressure measurement for all patients. Corneal ulcer size measured by photo slit lamp, location, duration, depth and margin of infiltrating, possible associated complications, and treatment were recorded. All patients underwent ulcer photography and measurement of cornea thickness. At initial presentation, all patients underwent corneal scrapping for Gram's stain, potassium hydroxide preparation (KOH wet mount), bacterial culture (blood agar and chocolate agar), and fungal culture (Sabouraud dextrose agar). Patients were started on topical antibiotics/topical antifungals based on the Gram's stain/KOH wet mount reports which were obtained immediately after the patient presented to us. There was no delay in starting topical medications in any of the patients {topical antibiotics like Ciprofloxacin (0.3%), Ofloxacin (0.3%), New generation fluoroquinolones: Moxifloxacin and Gatifloxacin, Fortified cefazolin 5% and tobramycin or gentamicin 2% &topical antifungals e.g Topical Natamycin (5%), Amphotericin B (0.15-0.3%)…etc} at hourly intervals to treat infectious corneal ulcers. All patients were prescribed topical atropine 1% give rest to the eye and pain medications are given as needed in addition to antibacterials and antifungals. Steroids are not used at any stage of treatment. Patients were followed up every third day and observed for signs of resolution of the corneal involvement and hypopyon. Those patients not responding to topical antibiotics/ topical antifungals for more than 2 weeks were considered as having non-resolving microbial keratitis involvement and advised to undergo CXL. Based on patients' agreement to have CXL, subjects were treated with CXL in addition to standard antimicrobial treatment.

2.1. *Surgical Technique of CXL*

All cases were done in EL-Nour Eye Center, Eye Care Center located in Assiut, by one doctor, From January 2019 To December 2020. Standard Dresden Protocol is used. First, Anesthetize the cornea with topical tetracaine of 0.1% drops 5 min before and just before the procedure. Eyelashes were isolated by a drape and a speculum. An 8 mm zone of corneal epithelium including all microbial infiltrates was removed using a thin blunt metal (Hockey knife), and then 0.1% riboflavin in dextran 500 20% drops were administered every three minutes for 30 minutes. Then cornea was exposed to UV-A rays (365 nm) in an optical zone of 8 mm for 30 minutes with an irradiance of 3 mW/ cm^2. During the procedure, moistened the cornea every 5 minutes with 0.1% riboflavin and tetracaine drops. After irradiation, a therapeutic soft contact lens was used and removed one day after placement. Patients were continued with the same topical medications that were being used before CXL according to culture sensitivity test and artificial tear eyedrops until epithelial healing was observed. Topical corticosteroids or NSAIDs were not prescribed after the crosslinking procedure. After treatment with CXL, the patients were examined once weekly to evaluate response to treatment, re-epithelialization, and time to resolve stromal infiltrate. Treatment was considered successful if rapid epithelialization with a decreased stromal infiltration within four weeks after CXL. If No sign of improvement or the ulcer gets worse after four weeks, it is considered a failure. Assessment of the efficacy of treatment by: **1)** Duration of ulcer healing: Photography before the start of treatment and four weeks follow-up. **2)** Recording signs of improvement (V/A, A/C reaction, ulcer: decrease in size and infiltration, epithelization, and increased vascularization). **3)** Patients had relief of symptoms (pain, redness, lacrimation, and photophobia).

2.2. *Follow up*

First postoperative day, once weekly for up to 3 months. This prospective interventional study was performed in correspondence with the Declaration of Helsinki and was reviewed and approved by the Assiut University Institutional Review Board. Written informed consent was obtained from all patients before enrollment in the study, following a discussion about the study's nature and the risks/ benefits of participation.

2.3. *Statistical Analysis*

Statistical analysis was done using SPSS version 22 (IBM SPSS Inc. Chicago, US) for windows 10. Nominal data were expressed as numbers and percentages while continuous data were expressed as mean, standard deviation, and range. Mann Whitney test was used to compare time to healing between bacterial and fungal infection. The level of confidence was kept at 95% and hence, the P value was significant if < 0.05.

3. Results

In all, 16 eyes of 16 patients including 10 men and six women with culture-proven non-healing microbial keratitis were included in the study. The mean age of enrolled patients was 56 ± 16.66 years with a range between 23 and 80 years. 62.5% of those patients were males and 6 (37.5%) patients were females. No previous eye surgery in 68.8%. Three patients had previous cataract surgery, one patient had a glaucoma operation and the other one had a history of tarsorrhaphy, tab. (1).

Table 1: Baseline data of enrolled patients

Descriptive Variables	Number of patients
• **Age (years)**	56 ± 16.66
• **Range**	23-80
• **Sex**	
Male	10 (62.5%)
Female	6 (37.5%)
• **Previous eye surgery**	
None	11 (68.8%)
Cataract	3 (18.8%)
Glaucoma	1 (6.3%)
Tarsorraphy	1 (6.3%)

Data expressed as frequency (percentage). N: number

3.1. *Ocular examination and characteristics of the ulcer*

In 9 patients (56.3%) the right eye was affected while the left eye was affected in 7 patients (43.8%). Seven (43.8%) and 9 (56.3%) patients had high and normal intraocular pressure, respectively. Ulcer Size range from smallest to largest size with a mean of 5.18 ± 1.27 mm. The ulcer was central in 13 (81.3%) patients, paracentral in 2 (12.5%) patients, and peripheral in only one patient. Vascularization was detected in 11 (68.8%) patients. Hypopyon was present in only 5 (31.3%) patients. Stromal Infiltrate was 1/3 depth of stroma in 4 patients (25%), range between 1/3 to 2/3 depth of stroma in 3 patients (18.8%), 2/3 stromal depth in 2 patients (12.5%), and >2/3 of stromal depth in 6 patients (37.5%) patients, tab. (2).

Table 2: Ocular examination and characteristics of the ulcer in the patients

Descriptive Variables	Number of patients
• **Affected eye**	
Right	9 (56.3%)
Left	7 (43.8%)
• **Vascularization**	11 (68.8%)
• **IOP**	
High	7 (43.8%)
Normal	9 (56.3%)
• **Site of ulcer**	
Central	13 (81.3%)
Paracentral	2 (12.5%)
Peripheral	1 (6.3%)
• **Size of the ulcer (range from smallest to largest size)**	5.18 ± 1.27
• **Depth (stromal infiltrate)**	
1/3	4 (25%)
1/3-2/3	3 (18.8%)
2/3	2 (12.5%)
> 2/3	6 (37.5%)
• **Hypopyon**	5 (31.3%)

Data expressed as frequency (percentage). N: number

3.2. *Type of infectious keratitis*

Seven (42.8%) patients had a bacterial infection in form of Staph. Aureus (three patients), Streptococcus species (two patients), and pseudomonas (two patients).

Fungal infection in seven patients (42.8%) in form of *Aspergillus spp* (four patients), *Fusarium spp*. (one patient) and candida species (one patient). Only one patient had a mixed infection and another patient had acanthamoeba, tab. (3) & figs. (1-3)

Table 3: Duration of therapy and type of infection among enrolled patients

Type of infection	Number of patients
• **Bacterial**	7 (42.8%)
Staph. Aureus	3 (18.8%)
Streptococcus species	2 (12.5%)
Pseudomonas	2 (12.5%)
• **Fungal**	7 (42.8%)
Aspergillus	4(24.5%)
Fusarium spp	2(12.2%)
Candida	1(6.1%)
Mixed	1 (6.3%)
• **Acanthamoeba**	1 (6.3%)

*Data expressed as frequency (percentage). **N**: number*

3.3. *Visual acuity baseline and follow-up*

Baseline visual acuity was in form of hand motion in 6 (37.5%) patients and bad perception of light (PL) in 5 (31.3%) patients. Follow-up visual acuity was in form of good PL IN 4 (25%) patients and 1m/60 in 3 (18.8%) patients. Other data of visual acuity. There was a significant improvement in the visual acuity during follow-up in comparison to baseline visual acuity (2.83 ± 0.70 vs. 1.93 ± 0.84; P< 0.001), tab. (4). The time of healing among enrolled patients was 28.67 ± 10.39 days with a range between 14 and 50 days. A patient with acanthamoeba infection required keratoplasty secondary to recurrence three months later. It was noticed that time to healing was significantly lower among bacterial infections in comparison to fungal infections (20.85 ± 5.55 vs. 35.57± 9.37 (days) (P< 0.001), tab. (5).

Table 4: Baseline and follow-up visual acuity

Descriptive Variables	Number of patients
• **Baseline visual acuity**	
6/60	1 (6.3%)
6/18	1 (6.3%)
Hand motion	6 (37.5%)
PL with Good projection	2 (12.5%)
PL with Bad projection	5 (31.3%)
No perception of light	1 (6.3%)
• **Follow-up visual acuity**	
6/60	1 (6.3%)
6/36	1 (6.3%)
6/18	1 (6.3%)
2/60	2 (12.5%)
1/60	3 (18.8%)
0.50/60	1 (6.3%)
Hand motion	2 (12.5%)
Good perception of light	4 (25%)
No perception of light	1 (6.3%)
• **Visual acuity (by logmar)**	
Baseline	2.83 ± 0.70
Follow up	1.93 ± 0.84
P value	< 0.001

*Data expressed as frequency (percentage), and mean (SD). **P value**: was significant if < 0.05.*

Table 5: Time to healing among enrolled patients (n= 16).

	All patients	Bacterial infection	Fungal infection
Time to healing (days)	28.67 ± 10.39	20.85 ± 5.55	35.57 ± 9.37*

*Data expressed as mean (SD). N: number. *indicates significant differences between thosewith bacterial and those with fungal infection*

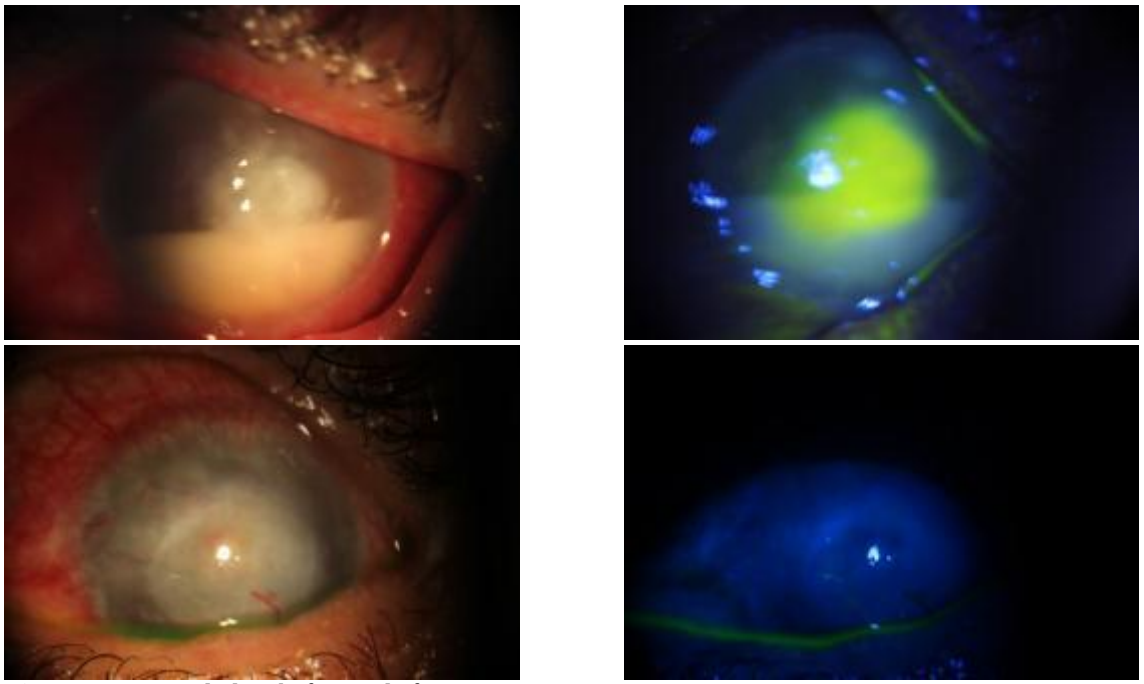

Figure 1: Bacterial ulcer before and after CXL

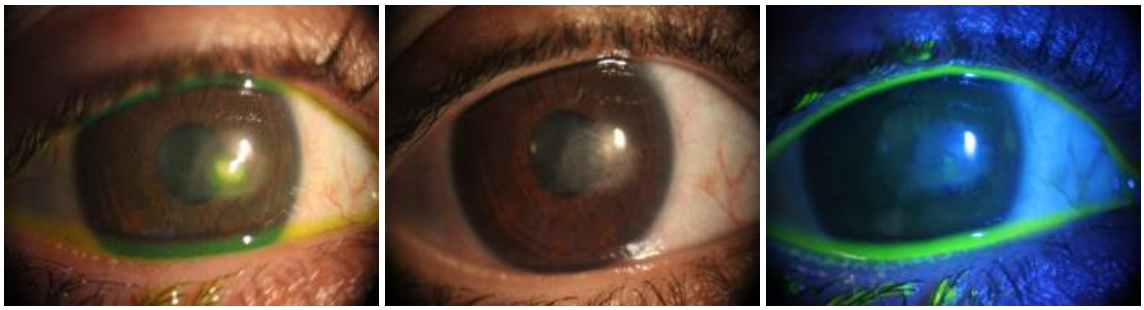

Figure 2: Acanthamoeba keratitis before and after CXL

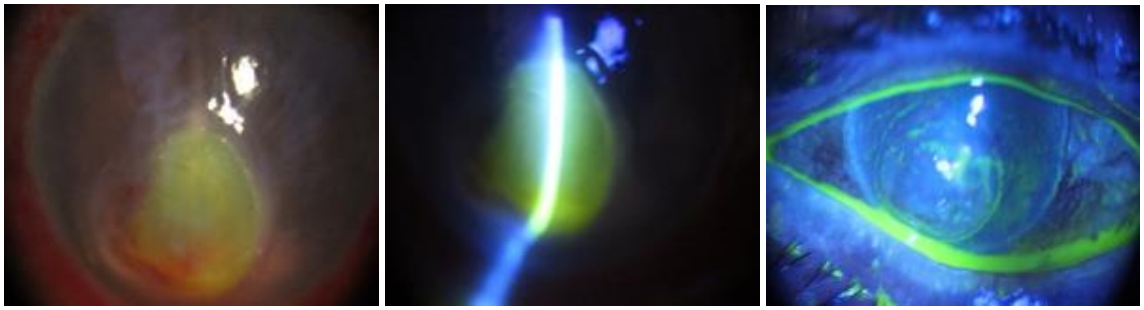

Figure 3: Fungal keratitis before and after CXL

4. Discussion

There are just few studies accessible because of the ethical problem of choosing patients to treat with CXL without antibiotic therapy influencing the outcome. In the majority of these studies, this treatment was selected for recurrent and unresponsive keratitis. We also saw that all patients who underwent CXL had a resolution of

pain on the first postoperative day. The possible explanation for this could be harm to the subepithelial nerve plexus by the riboflavin–UVA combination due to 'chemical denervation'. The reduction of corneal sensation in all patients post-CXL supports this hypothesis. All patients showed clinical improvement in ulcer characteristics and revealed a decrease in pain and other symptoms. These results are viable with other reports on the beneficial effect of CXL for resistant infectious keratitis [9-11]. In our review, the predominance of infectious keratitis was in the middle age group (Mean age = 56 ± 16.66) and among males (62.5%), which could be attributed to their greater involvement in outdoor activities, thus more prone to corneal injury with external agents. Similar observations were reported by other studies [12-14]. Contact lens wear was noted in just 6.3% of patients with microbial keratitis, however, contact lens wear was reportedly one of the major associated conditions in other studies [15-16], because of contact lens-induced hypoxia and hypercapnia of the cornea. The lesser incidence in our study could be explained because of the low financial level of the patients included and the low number of patients included. In the current study, the causative organisms were fungi & bacteria by an equal percentage =42.8%. Gram-positive bacteria were more common than Gram-negative bacteria, and S. aureus was the most commonly identified isolate, followed by P. aeruginosa & Strept. pneumonia. The incidence of fungal keratitis range from 6% and 56%. A hot, humid climate and an agriculture-based occupation of a large population make fungal keratitis more frequent than in Egypt. Aspergillus species were the most common fungi, involved in 24.5% of the fungal cases, followed by Fusarium spp. (12.2%) that agree with previous comparable studies in Egypt [17], Bangladesh, and India. S. aureus were the most common bacteria (18.8%) isolated in our patients. The same finding has been observed by others [14]. The spectrum of microrganisms accounting for microbial keratitis differs depending on geographic location, climate, and etiology. For example, gram-positive bacteria are predominant in temperate climate regions, whereas Gram-negative bacteria are prevalent in tropical regions. Pseudomonas spp. are associated with contact lens-related infections, whereas fungi are related to trauma caused by plants [14,18]. In our study, only one (6.25%) case of Acanthamoeba keratitis was reported, which is consistent with other studies of microbial keratitis in which the incidence of Acanthamoeba ranged from (0-8%) of the culture-positive cases [18]. However, it is much less than what was reported in a previous Scottish study (70%) [19]. Several articles describe the effect of UV radiation on microorganisms [20-21]. UV radiation is commonly used in operating rooms as a germicide. In 1960, it was shown that riboflavin exposed to UV inactivates RNA in several viruses. Recent studies have also described riboflavin as a photosensitizer that inactivates pathogens in plasma, platelets, and red blood cells [5,6]. Another study showed that CXL (UV-A and riboflavin) increases the resistance of the cornea against enzymatic lysis by microorganisms [22]. Theo Seiler's group reported that the CXL might be used in therapy-resistant cases to avoid emergency keratoplasty [23]. Similar results on using CXL as adjunctive treatment in medically resistant cases have been published by other authors [9,10,24]. In 2014, Shetty, et al. [25] reported that patients with deep stromal keratitis or endothelial plaque failed to resolve in their research. Hence, we evaluated the effectiveness of CXL in our patients with infective keratitis. We found that in patients with superficial corneal infiltrates involving only the anterior third or less of the stroma, there was a better response than in those cases with deeper infiltrates. CXL seems to be

a valuable adjunctive treatment in such cases because its mechanisms of action are different from antibiotics. The therapeutic effect of CXL in corneal ulcers could be related to its toxic action against pathogens and the increase in collagen resistance against enzymatic degradation. UV irradiation has antimicrobial activity and has been traditionally used for disinfection of blood transfusion products, drinking water, and air or surfaces. In a 2003 pilot study of patients with keratoconus, Wollensak, et al. [26] showed that transparency of the cornea, lens epithelial cell density, and intraocular pressure remain unchanged after CXL treatment, which means that corneal CXL with riboflavin has no serious side effects. In our study, there were no severe side effects in patients treated with CXL. The standard CXL protocol (30 min, 3 mW/cm^2) produces an effective treatment depth of approximately 250-300 mm. So, the infiltrate depth should be assessed before treatment. Treatment efficacy decreases with depth because the UV-A light is absorbed as it passes through the riboflavin-soaked cornea. The effect of CXL has been detected up to a depth of 300 mm of the cornea. Hence, there are concerns of damage to the corneal endothelium and crystalline lens in corneas less than 400 mm thick. However, in patients with corneal ulcers, the hazy/opaque cornea and the presence of corneal edema limit UV light penetration [27]. So, we are concerned about our study for those patients with corneal thickness > 400 mm. Several recent studies have confirmed the efficacy of the Dresden protocol when CXL is used to stop the keratoconus progression and treat corneal melting and infectious keratitis [28,29]. The use of CXL in the management of infectious keratitis is due to a good effect on the corneal melting block in combination with the antimicrobial properties of photoactivated riboflavin. Makdoumi, et al. [30] found extensive eradication of bacteria by riboflavin photo-activation using UVA (365 nm) in their in vitro study. Likewise, Schrier, et al. [31] reported the effectiveness of a combination of riboflavin and UVA exposure for 30 minutes against Staphylococcus aureus and Pseudomonas aeruginosa. Significant antibacterial action of UVA/riboflavin treatment was demonstrated by Martins, et al. [32] in their in vitro study that included methicillin-resistant S. aureus, multidrug-resistant P. aeruginosa, and drug-resistant Streptococcus pneumonia. In our study, we observed a rapid improvement in symptoms and found that the sizes of epithelial defects and areas of infiltrates were smaller 7 and 14 days after starting treatment. One patient with mixed keratitis required a Conjunctival flap but other patients' eyes healed rapidly without any complications. By shortening the course of treatment, CXL can reduce the toxic effects of topical antibiotics on the cornea and prevent healthcare-associated infections. Compared with bacterial infections, fungal infections often penetrate more deeply and may be more dependent on the body's cellular immune response for eradication, so wiping out host immune cells in the anterior cornea may be more problematic with fungal infections. There is less robust evidence to support the use of CXL in treating filamentous fungal keratitis. In vitro CXL alone has not been shown to inactivate fungus, although one in vitro study did find CXL plus amphotericin to improve inhibition of fungal pathogens over amphotericin alone [32,33]. Kashiwabuchi, et al. [33] and Sauer, et al. [34] found that CXL was ineffective against candida Albicans. Unlike Galperin, et al. [34] demonstrated a significant effect of CXL against Fusarium solani in their animal model. Said, et al. [36] and Bamdad, et al. [37] reported the time to complete corneal healing (or treatment duration), which was defined as complete re-epithelialization and disappearance of infiltration and hypopyon. In our study,

it was noticed that CXL was effective against fungal cases and resulted in complete healing leaving a scar in all cases but also noticed that time to healing was significantly lower among bacterial infections in comparison to fungal infections 20.85 ± 5.55 vs. 35.57± 9.37 days. The unspecific presentation and rarity of acanthamoeba keratitis are the primary factors in delaying the diagnosis. The delay ranged from 7 weeks to 12 months. Besides, corticosteroid therapy if initiated can result in disease progression. The annual incidence of Acanthamoeba keratitis among contact lens users is increasing, which could be due to a lack of proper hygiene and contamination of lenses. Several trials have recently shown the effectiveness of UV light in treating AK resistant to medication [38]. Other studies are less encouraging for the management of Acanthamoeba keratitis because no antitrophozoite activity and no efficacy of CXL in decreasing the intensity and severity of Acanthamoeba keratitis were proven neither by Berra, et al. [39] in an animal model nor by Kaschiwabushi, et al. [40] in both an in vitro study and an animal model. In our study, the only case with AK showed improvement in all symptoms and complete healing after 2 months from cxl. Unfortunately, recurrence of infection occurs after 3 months post-CXL, and penetrating keratoplasty is recommended.

Conclusion

CXL appears to be an effective procedure in treating non-resolving microbial keratitis with superficial stromal involvement. The most promising results published so far are for keratitis especially when the germs do not involve the posterior stroma and in cases of impending perforation. CXL can be an effective adjunctive treatment in the management of resistant microbial keratitis. The sterilizing effect of CXL, its anti-angiogenic action, and its ability to induce tissue remodeling could improve the survival rate of a corneal transplant in an inflamed and vascularized bed. CXL may also represent a valid additional tool to the standard treatment in cases in which emergency corneal transplantation is required. Additional randomized clinical trials are needed to support encouraging results on the use of CXL in the management of microbial keratitis.

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