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Review Article

Research Progress on Corneal Collagen Cross-Linking for Corneal Ulcerative Keratitis

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Abstract: Corneal collagen cross-linking (CXL) is a photochem therapy for keratoconus, keratectasias and infectious keratitis. Corneal collagen fiber infiltrated with riboflavin, a photosensitizer which generates reactive oxygen species when activated by UVA, was induced to form the cross-links in corneal stroma. The purpose of this paper is to review the basic principle, therapeutic potential of corneal ulcerative disease, existing evidences from laboratory and clinical studies, and relevant limitations, then put forward the advantages of CXL in the treatment of corneal ulcerative disease, and thus provide more possibilities for the treatment of the disease.

Keywords: Corneal Collagen Cross-Linking (CXL), Ultraviolet Light A (UVA), Riboflavin, Ulcerative Disease

1. Introduction

Corneal ulcerative keratitis (corneal ulcer disease) includes infectious keratitis and non-infectious keratitis caused by ulcerative diseases. Infectious keratitis is a common ocular disease caused by a variety of pathogens, including bacteria, fungi, viruses, and Acanthamoeba parasites. Causes of non-infectious keratitis include corneal alkali burns, immune factors, infections, and eye surgery. If corneal ulcer is not treated in time, it may cause severe visual impairment and blindness. At present, the clinical treatment of infective keratitis mainly adopts antibiotic therapy, but the problem of repeated attack or drug resistance due to the disease is difficult to treat. The treatment of non-infectious keratitis is mainly treated by corticosteroids and immunosuppressants, but it is difficult to treat because of the problems of prolonged illness, repeated attacks and no targeted drugs. Severe infected patients eventually need corneal transplantation. The application of corneal collagen cross-linking has brought a new treatment for corneal ulcer disease.

2. The Principle of Corneal Collagen Cross-Linking

Corneal collagen cross-linking (CXL) is a new method for the treatment of corneal dilatation (*1*). The application of cross-linking technology has a long history, and has been widely used in industrial and biological engineering fields, for example, the hardening of polymeric materials, the artificial heart valves and the stability of dental materials. The basic principle is that riboflavin is used as sensitizer and activated riboflavin as triplet under ultraviolet radiation with wavelength of 370nm to produce reactive oxygen species, which further interact with a variety of molecules to form covalent bonds between the amino groups of adjacent collagen fibers. This results in increasing the resistance of collagen fibers to collagenase and the test results of stress-strain variables (i.e. bio-mechanical strength) (*2*). Riboflavin as a sensitizer can not only increase the absorptivity of ultraviolet rays, but also induce the cross-linking of collagen fibers, protect against the

infiltration of UVA in the underlying tissues, and avoid the damage of deep ocular tissues. The 360-370nm wavelength UV radiation should be used to ensure that all structures are exposed below harmful levels.

3. Pathogenesis of Corneal Ulcer

The basic pathological process of corneal ulcer is corneal collagen degradation. The decomposition of corneal collagen can be divided into tissue collagenase produced by corneal innate cells, immune cells and collagenase produced by pathogenic microorganisms. The mechanisms of these two collagenases are different. Collagenase from pathogenic bacteria can completely decompose collagen molecules into small peptide chains consisting of three amino acids. However, tissue collagenase secreted by corneal stromal fibroblasts and fibroblasts could only degrade the -Gly-Ileu-binding site in collagen fibers. While only one -Gly-Ileu-structure was found in collagen fibers per share, it requires other collagenases to work together to completely degrade collagen molecules (*3*).

Most of the extra-cellular matrix of the cornea of normal human eyes is collagen fiber. When the cornea is inflamed, many kinds of enzymes in the microorganism and host are synthesized and secreted, which can destroy the tissue. Some of them have been identified as collagenase and a variety of proteases. Studies have shown that corneal collagen melt is caused by matrix metalloproteinase (MMPs), which is mediated by and acts on corneal stromal collagen through cellular activation (*4*). The main function of MMPs is to degrade extra-cellular matrix and basement membrane components. All activated MMPs can be inhibited by tissue inhibitor of metalloproteinases (TIMPs), and the expression of MMPs is regulated by many cytokines, such as leukocyte interleukin-1 (IL-1), transforming growth factor- β (TGF-β) and so on. In addition, neutrophils enhance the degradation of collagen by corneal fibroblasts, which is caused by the stimulation of MMPs synthesis and the release of IL-1 by neutrophils (*5*). At the same time, the pathogenic microorganisms secreted toxins, which aggravated the damage to the cornea. (*6*) For example, streptococcus produces intracellular hemolysin that speeds up intracellular enzyme activity and destroys the cornea, and aflatoxin produced by molds inhibits the activity of DNA and RNA polymerase and destroys protein synthesis. As a result, corneal ulcers are produced through the following mechanisms: 1) The imbalance of the regulation of collagenase and its activator and inhibitory factor; 2) Related cytokine regulation accelerating corneal matrix melt; 3) The destruction of corneal stroma by toxins secreted by pathogenic microorganisms.

4. The Possibility of CXL in the Treatment of Corneal Ulcer

1. The hardness of cornea increased after CXL, which enhanced the antienzymatic activity of protease and collagenase. The ocular response analysis software used by Spoerl et al. (*7*) found that the bio-mechanical properties of cornea were changed after CXL. Studies have shown that (*8*) pepsin, trypsin and collagenase are used to digest the cornea of rabbit eyes after CXL. The results show that CXL can enhance the bio-mechanical characteristics of corneal tissue and make its structure more firm and stable. It improves the ability of cornea to resist digestive enzymes.
2. After CXL, the structure of the corneal tissue is changed, the target site of collagenase acting is destroyed, and the anti-enzyme reaction is increased. Spoerl et al. (*9*) reported the clinical efficacy and bio-compatibility of riboflavin/UVA cross-linking in human cornea. After CXL, the new chemical bonds of cornea were induced and the reaction to enzyme degradation was increased. It is inferred that collagen fibers may form new covalent bonds and form a new molecular space structure after CXL, which affects the destruction of corresponding molecular sites by collagenases. Thus, the ability of cornea after UV-riboflavin cross-linking treatment against various enzyme digestion was significantly enhanced.
3. After CXL, the infiltration of inflammatory cells in corneal tissue is reduced, thus the stimulating effect of inflammatory cells on collagenase synthesis is weakened, the cytokines released by neutrophils are decreased, and collagen degradation mediated by corneal fibroblasts is reduced. Zhao Dongxu et al. (*10*) imitated alkali chemical burns on rabbit eyes. After CXL treatment, the corneal melt in the experimental group was obviously superior to that in the control group from pathological and immunohistochemical results. They suggest that CXL can increase corneal tolerance to hydration, reduce corneal edema and protect deep corneal tissue, so the infiltration of inflammatory cells in the deep corneal stroma is reduced and collagen fibers are arranged neatly.
4. The combined application of riboflavin and ultraviolet rays can make the activity of pathogens more inactivated. The effect is the result of DNA damage at the molecular level. Riboflavin/UV induces changes in guanine bases on DNA, thereby eliminating or inhibiting the propagation of pathogens (*11*). Sauer's research team designed agar plates containing multiple treatment and control groups. (*12*) In the treatment group with amphotericin B cross-linked with UV riboflavin, the fungal growth was significantly inhibited. However, the results of one treatment alone, two ways of treating the experimental group and the control group without any treatment showed that there was no significant inhibition on the growth of fungi, which indicated that UVN crosslinking could enhance the anti-fungal effect of amphotericin B.
5. Ultraviolet light A (UVA) has anti-microbial effect. Some studies have found that ultraviolet A riboflavin

irradiation can reduce pathogens in blood products such as platelets, fresh frozen plasma, red blood cells, and ensure that blood products are safe and effective during blood transfusions. It not only reduces the infection through blood, but also proves the anti-microbial effect of ultraviolet A riboflavin cross-linking technology.

6. The CXL technology can enhance corneal repair. Hao Zhaoqin et al. (*13*) found that more fibroblasts were found between corneal collagen fibers after CXL. It may be that CXL stimulated the proliferation of fibroblasts, increased the number of collagen fibers, and accelerated corneal repair. Thus, the mechanical stability of corneal stroma is further enhanced.
7. The study also found that riboflavin could reduced nociceptive response of corneal nerves that decrease pain and reduced tendency to generate vascularization (*14*). Shetty et al. confirmed that in patients with refractory advanced microbial keratitis, pain was significantly reduced after treatment with CXL (*15*).

5. Clinical Application of CXL

5.1. Corneal Ulcer Caused by Infectious Keratitis

Igal et al. (*16*) reported that a young patient diagnosed with fungal keratitis had no significant improvement after antifungal therapy. A couple of days later, a corneal melting process was observed and as the corneal melting continued, it was decided to try treatment with CXL. The patient underwent a CXL treatment and shortly thereafter, the abscess shrank, with significant improvement in symptoms and vision.

Khan et al. (*17*) present the first 3 cases of Acanthamoeba keratitis (AK), unresponsive to medical treatment, that were successfully treated with a novel adjunctive therapy using UVA and riboflavin (B2). Two patients with confirmed AK and one patient with presumptive AK. There was no obvious effect on these 3 patients after routine multi-drug therapy and anti-infection therapy, so they were treated with two courses of ultraviolet light A riboflavin mediated CXL. All patients in these series showed a rapid reduction in their symptoms and decreased ulcer size after the first treatment session. The symptoms did not improve significantly after 1 to 3 weeks, and then continued the second course of treatment to strengthen and consolidate the curative effect. Finally, the symptoms of the patients were obviously alleviated, and the corneal ulcers formed scar tissue. It can be seen that the prognosis of Acanthamoeba keratitis treated with UVA/riboflavin mediated CXL is better than that of conventional anti-infective therapy.

In 2012, Panda et al. (*18*) evaluated the therapeutic effect of photo-activated riboflavin (PAR) for treating refractory corneal ulcers, which included 7 cases (7 eyes) with infectious keratitis, presented with a gradually deteriorating, vision-threatening, corneal ulcer, despite intense antimicrobial therapy, were treated with PAR. In all cases, the progression of corneal melting was halted after PAR treatment. Emergency keratoplasty was not necessary in any of the 7 eyes presented. More importantly, all the ulcers were healed without significant vascularization. As a result, they concluded that PAR is a promising option for treating patients with therapy-refractory infectious keratitis to avoid emergency keratoplasty and should be considered as a potential adjuvant therapeutic tool in such eyes.

A prospective comparative clinical trial assesses the efficacy of CXL versus conventional therapy. 40 patients (40 eyes) with advanced infectious keratitis and coexisting corneal melting. (*19*) Twenty-one patients (21 eyes) underwent PACK-CXL treatment in addition to antimicrobial therapy. The control group consisted of 19 patients (19 eyes) who received only antimicrobial therapy. CXL with photoactivated riboflavin did not shorten the time to corneal healing. However, the complication rate was higher in the control group, whereas there was no incidence of corneal perforation or recurrence of the infection in the PACK-CXL group. These results indicate that CXL may be an effective adjuvant therapy in the management of severe infectious keratitis associated with corneal melting.

Current clinical studies suggest that CXL can effectively prevent corneal stroma melting. The ability to remove or inhibit the growth of some pathogens, including cases of keratitis caused by a variety of organisms, such as bacteria, fungi, parasites, and Acanthamoeba cases. CXL can also be used as an adjuvant treatment for severe drug-resistant infectious keratitis, an additional treatment option before corneal transplantation, which can avoid or postpone corneal transplantation.

5.2. Corneal Ulcer Caused by Non-Infectious Keratitis

Non-infectious ulcers (*6*) could occur alone, such as Mooren corneal ulcers, could be associated with various collagen vascular diseases, and could damage to the integrity of ocular surface tissues, such as corneal alkali burns, exposed keratitis, neurotrophic keratitis, and persistent epithelial defect caused by eye surgeryetc, etc. At present, the application of CXL in non-infectious cornea research is at the animal experimental stage. Zhao Dongxu (*10*) studied the effect of CXL therapy on the corneal stroma melting of rabbit cornea after alkali burn. The results showed that the normal control group did not change. In the model control group, 6 eyes corneas were corneal melting and 2 eyes were corneal perforation. In the CXL group, 1 eye was corneal melting after 23 days treatment, but no cornea perforation occurred. Pathological examination showed that the degree of corneal edema, corneal collagen fiber destruction and inflammatory cell infiltration in the CXL group were less than those in the model control group. The results of immunohistochemistry showed that the corneal collagen fibers of the CXL treatment group were arranged more neatly than the model control group, and the deep corneal tissue had little effect.

6. Limitations of CXL in Corneal Ulcer

6.1. Research and Experimental Design

1. The evidence is weak. Most of the evidence is descriptive in the form of case reports and case series, but not enough in terms of sample size, or lack of randomized controlled trials to compare the same severity caused by the same pathogen.
2. Infectious corneal ulcers include bacteria, fungi, herpes simplex virus and Acanthamoeba infection. While the number of bacterial keratitis cases is high enough, the literature provides only limited experience for infection by other organisms. The results of CXL in the treatment of fungi and Acanthoamoeba do not provide clear evidence to support or oppose these cases (*20*); In the case of herpes keratitis, CXL may lead to significant corneal thinning or even melting. (*21*)
3. There are few studies on non-infectious ulcers, which are only at the animal experimental stage. The design of the model control group is difficult, which hinders the experimental research, such as the design of Mooren corneal ulcer, collagen vascular disease and other diseases.
4. The reports describe cases with variable disease severity from very small superficial infiltrate to moderately severe keratitis with stromal melt. However, the severity of different levels of keratitis lacks a standardized assessment and therefore cannot provide a clear definition. (*22*)
5. The term "stromal melt" is vague, and the data on residual stromal thickness or extent of melt are not provided. Therefore, one cannot make conclusions on its safe use in the presence of severe stromal loss and thinning. Further, the stromal loss in corneal infection is not uniform, and the possibility of finding areas of significant stromal loss and normal thickness within the same cornea cannot be ruled out. (*23*)

6.2. Experimental Safety

A large number of published investigations have evaluated the security of CXL technology. The researchers concluded that no corneal morphological abnormalities were observed after CXL treatment, and endothelial cell density and macular foveal thickness remained unchanged. (*24*) Subsequent study (*25*) reported no change in lens density and macular fovea thickness after 12 months CXL treatment, while the third study (*26*) confirmed that retinal morphology was not significant after CXL treatment.

It should be noted that patients with keratoconus exhibit corneal stroma thickness less than 400um, and they can obtain satisfactory vision by wearing glasses or contact lenses. The current CXL treatment regimen is prohibitive for surgery where the corneal thickness is less than 400um. Nevertheless, it has also been proposed to change the alternative treatment plan for thin corneas. (*27, 28*) They used low osmotic riboflavin solution with satisfactory results. However, corneal thickness less than 400um is beyond the scope of the Dresden protocol, the surgical risk is greater, and the incidence of complications may be higher. For example, Kymionis et al. recorded a significant decrease in endothelial cell density. (*29*) Therefore, in order to reduce unnecessary injury, the thin cornea below 400um must be excluded before operation, and UVA irradiation dose should be checked. In addition, the most important exclusion criterion is penetration depth. If its depth exceeds 250um, UVA riboflavin will have a higher risk of endothelial cell loss and the effect of treatment will be lower. Because fungal infections are deeper than bacteria, anti-fungal treatments are less effective and more dangerous than bacteria. (*22*)

6.3. Complication

6.3.1. Effect of Riboflavin/UVA Irradiation

Due to the effect of riboflavin, the absorption of UVA in the cornea increased greatly during the CXL process, but the UVA transport through the cornea was only 7%. The radiation absorption of the intraocular structure below the corneal stromal layer is greatly reduced. All residual UVA energy is less than 1 J/cm, which is similar to the solar absorption energy accepted for daily outdoor activities. (*30*) CXL has no effect on apoptosis, but it may affect the activity of corneal stromal cells, and even have a certain impact on the local immune response of pathogens. (*31*)

6.3.2. Effects of Epithelial Removal on Corneal Healing

Since epithelial excision is associated with epithelial cell derived cytokines (such as interleukin -1), these cytokines stimulate keratinocytes to produce chemokines, and chemokines attract inflammatory cells (*32*). There are also some patients with ocular surface disease and specific diseases. These causes can lead to delayed epithelial regeneration, infectious/non-infective corneal stroma infiltration, etc.

Mild corneal haze was seen in almost all patients in the early postoperative period, but most of them gradually subsided within one year after surgery. Corneal haze is mostly located in corneal stroma, and the depth of corneal opacity can reach 60% of corneal thickness. A few patients may have severe stromal opacity that persists for more than one year, corrected visual acuity, and even permanent corneal stroma scar.

7. Conclusions

To sum up, in recent years, basic and clinical studies have shown that ultraviolet A combined with riboflavin mediated CXL technique plays an inhibitory and delayed role in the treatment of corneal ulcer disease. CXL provides new therapeutic ideas for corneal ulcers caused by various causes, reducing the need for corneal transplantation, which will be a new breakthrough in the treatment of corneal diseases. However, the possibility of CXL in the treatment of corneal ulcer disease has not been fully concluded, many of which are based on the conjecture or inference of the experimental

observations. At the same time, there are some limitations in the experimental design, and long-term clinical observation is needed for the safety of the experiment and postoperative complications.

8. Future Directions

At present, the Dresden protocol is commonly used as setting for CXL (3mw/cm2 and 365nm wavelength for irradiation of the cornea for 30 minutes), which is mainly used for keratoconus and post-operative corneal dilatation. Now, there is growing evidence shows that it can be determined whether it can bring better results to patients by modifying the irradiation time, parameters of irradiance energy, and even the type of chromophore. There are different approaches to the treatment of infectious keratitis in laboratory and clinical studies, some studies have shown that there is an accelerated method that uses 18mw/cm^2 for 5 minutes, or even 36mw/cm^2, irradiation for 5 minutes, can be observed high bacterial kill rate. (*33*) The use of photoactivated chromophore for infectious keratitis-corneal collagen crosslinking (pack-cxl) is better for the treatment of superficial infiltration and early ulcers. As microbes become more resistant to antibiotics, new treatments will be needed to replace them, so CXL may be a promising new alternative in the future. We need more research, especially the use of randomized controlled trials to assess the authenticity of CXL in the treatment of corneal ulcer disease. We also need to work out the protocol of consistency and propose the best treatment scheme and the best intervention time. The safety and complications of the treatment also need further experimental research and long-term clinical observation, thus providing more possibilities for CXL in the treatment of corneal ulcerative keratitis.

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