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ORIGINAL RESEARCH

Validation of different protocols of crosslinking and quality of vision in an experimental invitro model of eye bank corneas

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ABSTRACT

Background: Corneal collagen cross-linking (CXR) of human collagen is a physiological process to increase the mechanical and chemical stability of corneal. One major disadvantage of the CXR procedure so far is the long total treatment time of 1 hour including a soaking time of 30 minutes for the riboflavin solution and an illumination time of 30 minutes for the UV light. Therefore, to address the patient comfort, and the surgeon's efficiency, a shorter CXR procedure would be desirable. There are aims to shorten the soaking time by using a different protocol to apply the riboflavin and to shorten the illumination time by increasing the illumination intensity though maintaining the same total applied energy. Treatment time could be shortened to as low as 2 minutes by delivering a higher energy dose of 45Mw/cm2 through various new devices available now. But this accelerated procedure has not been validated yet. **Materials and Methods:** An experimental in vitro study was carried out on 40 eye bank corneas with 10 corneas as control, without cross linking,10 corneas underwent conventional CXL procedure with exposure to 3 Mw/cm2 of Ultraviolet(UV) light for 30 minutes, 10 corneas exposed to 9 Mw/cm2 of UV light for 10 minutes and 10 corneas exposed to 18 mw/cm2 of UV light for 5 minutes after debridement of cornea and soaking in riboflavin for 30 minutes. A test cell was fabricated to allow a differential pressure in range 15-20 mm Hg to be applied on the back side of test cornea while keeping it immersed in balanced salt solution, and images were acquired with polarizing light and set of lenses and camera. **Results and conclusion:** The results were in the form of images. The amount of stress decreases and the biomechanical strength increases with increase in irradiance time due to longer effect of UV exposure. The results were inconclusive regarding the effect of various protocols on optical quality of cornea.

Key words: Corneal collagen cross linking, birefringence, UV irradiation, Shack Hartmann wavefront sensor.

INTRODUCTION

The primary aim of this experimental study was to assess the effectiveness of new, accelerated protocols of crosslinking by comparing them to the standard Dresden protocol and to make recommendations regarding which protocol produces the maximum increase in strength of the cornea.

Cross-linking refers to a process of increasing intermolecular bonds. Corneal collagen cross-linking (CXR) is achieved using two components, riboflavin, a natural photosensitizer, present in humans and Ultraviolet A (UVA) light. CXR Works by creating additional chemical bonds between the corneal collagen fibrils in the corneal stroma by means of highly localised photopolymerisation while minimising exposure to surrounding structures of the eye.

This procedure was first developed by Prof. Theo Seiler, Prof. Wollensak and Prof. Eberhard Spoerl in 1997 at the university of Dresden in Germany. CXR alters the biomechanical properties of the cornea, resulting in an increase in the tensile strength of

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collagen fibrils. Though slight flattening of the cornea may be produced, it stabilizes the corneal curvature and prevents further steepening and bulging of corneal stroma, resulting in arrest of progression of keratoconus.

Riboflavin is a potent producer of oxygen radicals, especially singlet oxygen, though, the highest concentration of riboflavin is reached in the anterior stroma, however exposure of the riboflavin for 20-30 minutes produces sufficient concentration in the posterior stroma too. It is estimated that the stiffness of keratoconic corneas is 70% that of a healthy cornea. There is 328.9% increase in corneal rigidity following CXR. This increase in biomechanical rigidity after CXR is probably caused by an increase in collagen fiber diameter and due to interfibrillar and intrafibrillar covalent bonds.

One major disadvantage of the CXR procedure so far is the long total treatment time of 1 hour including a soaking time of 30 minutes for the riboflavin solution and an illumination time of 30 minutes for the UV light. There are aims to shorten the soaking time by using a different protocol to apply the riboflavin and to shorten the illumination time by increasing the illumination intensity though maintaining the same total applied energy. Treatment time could be shortened to as low as 2 minutes by delivering a higher energy dose of 45Mw/cm2 through various new devices available now. But this accelerated procedure has not been validated yet. Though it has been shown in ex vivo experiments that the biochemical stiffening effect of corneal tissue with 10mW/cm2 (illumination time of 9 minutes) is equivalent to the standard protocol.

The macroscopic in vitro experiments can be classified into two common methods viz., strip extension tests and inflation tests. Strip extension tests are more frequently used due to its simplicity and more reliable in terms of measurement of strain and stress because of known geometry, but are destructive and different from in vivo condition.

Young's modulus, which is used to calculate the stress induced in cross linked cornea at 10% strain, which is far beyond the in vivo physiological value. Above all, experiments have been done to validate the different irradiation protocols on porcine corneas, while human corneal data is still missing.

On the other hand, inflation tests are performed on intact corneas, changes in intraocular pressure can be simulated. Calculating stress and strain is complex due to the asymmetric geometry, anisotropic material properties and complex loading scenario. Due to photoelasticity, upon the application of stress, cornea exhibit the property of birefringence. The magnitude of the refractive indices at each point in the material is directly related to the state of stresses at that point. Information such as maximum shear stress and its orientation are available by analyzing the birefringence.

Nyquist discussed the stress-induced birefringence of the cornea and used stress-dependent dispersion of birefringence for IOP measurement. , While Ichihashi et al. using lenses, produced a polarization pattern similar to that of the in vivo cornea, and by performing computer simulations discussed the stress of the cornea in vivo. In addition, phase stepping imaging polarimetry was used by Jaronski and Kasprzak to measure birefringence of the human cornea in vivo and in vitro.

Hence, any change in strength of mechanical property of cornea under constant force should exhibit different birefringence pattern. It is also important to see the effect of crosslinking on optical quality of cornea as the relationship between birefringence change and a changé in optical quality of cornea has already been established. Measurement of wavefront aberrations, using a Hartmann Shacks sensor was used in keratoconus patients by Maeda et al., but there is no comparative data on the optical quality of cross linked and normal corneas

MATERIAL AND METHODS

The study was conducted at the Cornea & Anterior Segment Services located at KAR campus and biophysics laboratory of L V Prasad eye institute, Hyderabad. Human donor corneas obtained from the eye bank (with prior permission to conduct research, from donor relatives if not suitable for transplantation) obtained from Ramayamma international eye bank at L V Prasad eye institute, Hyderabad

Inclusion criteria

All donor corneas with wide scleral rim, with pachymetry between 400-650 microns which were unsuitable for transplantation were included.

Exclusion criteria

Corneas without scleral rim or with small scleral rims, with dystrophic or degenerative changes, with scars or evidence of prior corneal surgical procedures or from donor with a known history of ectasia were excluded from study.

Sample size

Study was done on 40 corneas (which were not suitable for transplantation) divided into 4 groups with 10 corneas as control (not cross linked), 10 corneas underwent conventional CXL procedure with exposure to 3 Mw/cm2 of UV light for 30 minutes (after exposing to 0.1% riboflavin following debridement of epithelium), 10 corneas exposed to 9 Mw/cm2 of UV light for 10 minutes and rest 10 corneas exposed to 18 mw/cm2 of UV light for 5 minutes. It was an experimental in vitro study on eye bank corneas

Methodology

After mechanical debridement of corneal epithelium over a zone of 8 mm, 0.1% Riboflavin drops with

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20% dextran was instilled onto the cornea at a raté of 1 drop/2 minutes for 30 minutes. Then, the cornea was exposed to UV irradiation at 370nm, instillation of 0.1% Riboflavin drops was continued during irradiation. In all the three groups, UVA irradiation of total equivalent energy of 3mw/cm2 for 30 minutes, 9mw/cm2 for 10 minutes and 18mw/cm2 for 5 minutes respectively is to be used.

With the development of Shack-Hartmann wavefront sensor (SHWS) and improvement in full field stress measurements using photoelasticity, It has become possible to quantify the changes in birefringence and optical aberrations.

A ten step phase shifting algorithm with a set of equations were used in this study, in which by taking ten phase shifted images, rotation and retardation at any point can be determined. The first four images were taken with the plane configuration for evaluation of the isoclinics (rotation θc) and later 6 images captured with the circular configuration for evaluation of the isochromes (retardation δc). The phase stepping was achieved by rotating the polarizer, analyzer and quarter wave plates by predefined angles.

The Tenets of the declaration of Helsinki protocol has been followed for handling the corneal tissues. The hydration and thickness of the cornea was assumed to be constant during the experiment. A test cell was fabricated to allow a differential pressure in range 15-20 mm Hg to be applied on the back side of test cornea while keeping it immersed in balanced salt solution. The test cell assembly (Figure 1) consisted of two polycarbonate discs, each with a 10 mm aperture in center fitted with an optical window. To allow quick replacement of cornea in the assembly, it is pasted on a 10 mm punched replaceable plastic film using cyanoacrylate glue. The plastic film is aligned to the optical axis and it is sandwiched between the two discs using a rubber seal. A gasket applied enough pressure to the discs to ensure seal. Once the pressure conditions were satisfied, the assembly was correctly aligned on its optical mount to ensure the alignment of optical axis (figure 2).

Figure 1 Two polycarbonate discs with optical windows. One has two tubing connected for building up differential pressures and accurate measurements

With cornea glued in the centre of this test cell (figure 3) balanced salt solution (BSS) will be filled on both the anterior and posterior side of the cornea. The refractive indices of cornea and BSS are comparable, so the wavefront of the light passing through the cornea change only due to the changes in the stress induced birefringence. To modulate the IOP, the posterior side of the cornea mounted in test cell is connected to a liquid column filled with BSS. The IOP i.e. the pressure on the posterior side can be manipulated by changing the height of the water column. A manometer tube connected to the test cell indicates the pressure inside the posterior chamber of the test cell. This test cell with cornea mounted on it, was then kept in the path of the scanning beam for photoelasticity and wavefront imaging. For simultaneous photoelasticity and wavefront imaging using a 633nm laser a polariscope capable of plane and circular configuration was built with a Shack Hartmann wavefront sensor (SHWS) coupled to it. By removing the quarter wave plates it is possible to switch from circular to plane configuration of the polariscope (figure 5).

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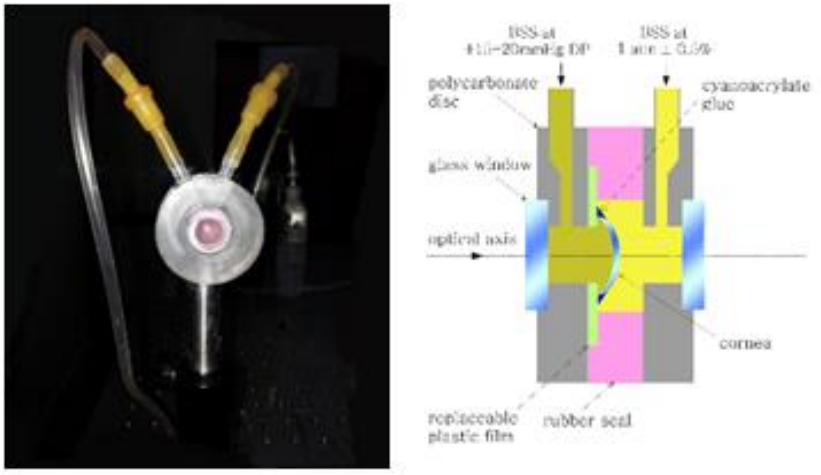

Figure 2 Real front view of test cell assembly and schematic cross section of test cell assembly

Figure 3 Cornea glued on replaceable plastic film and the experimental set up

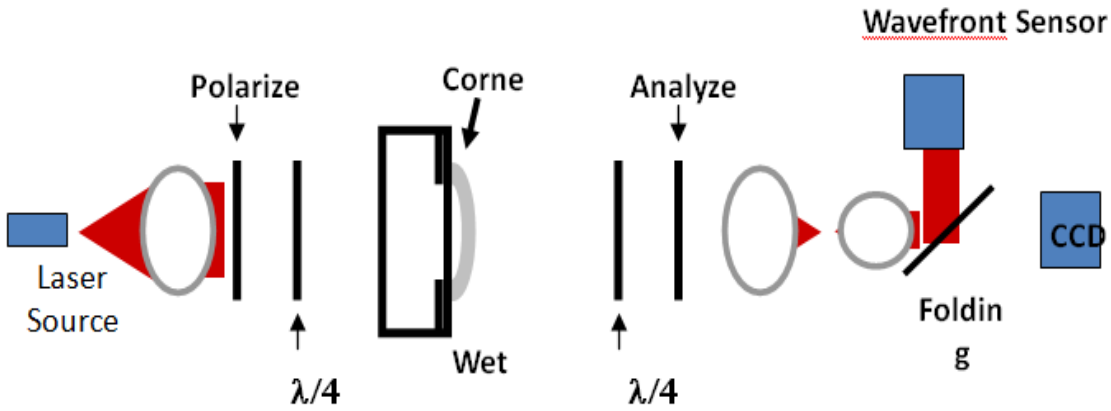

Figure 4 Polariscope equipped with shack-Hartmann wave front sensor

The images were unwrapped with another MATLAB program (Matlab 2009a, Mathworks Inc) based on quality guided phase unwrapping algorithm. Finally, the unwrapped images were smoothened by the robust outlier algorithm. Measured total birefringence was used for analysis with an assumption that component of material induced birefringence remained fixed for the experiment.

Statistical analysis

All statistical analyses were performed using the R software (version 2.12). The stress induced birefringence in human corneas of different groups were compared using the one-way ANOVA, and normalized cross correlation for image comparison (table 1). Normality of the data was checked using Shapiro-Wilk test. A p value of 0.05 or less is considered as statistically significant.

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Table 1: Comparison of the obtained Average Normalized and standard deviation values between same and different groups.

Group I	Group II	Normalized Values	
		Average	Standard deviation
5 min	5min	0.919711	0.06081
10 min	10 min	0.938378	0.047271
30 min	30min	0.874376	0.054737
5min	10 min	0.884763	0.020764
10 min	30 min	0.856271	0.021411
30 min	5min	0.875443	0.023509

RESULTS

The results were in the form of images. Hence comparing the magnitude of change is a robust method of evaluation. The colour represents the intensity of stress, with red colour meaning higher and blue means lower. First four images were taken with the plane configuration (figure 6) for evaluation of the isoclinics (rotation θc) and later captured six with the circular configuration (figure 7) for evaluation of the isochromes (retardation δc). The phase stepping was achieved by rotating the polarizer, analyzer and quarter wave plates by predefined angles. wave plate and analyzer are labeled as α, ξ, η, β, respectively.

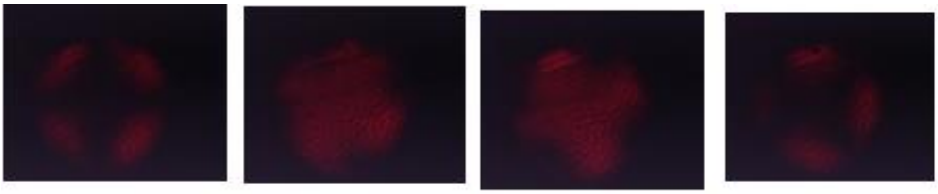

Figure 6 four images at plane configuration

Figure 7 six images at circular configuration

Figure 8 plot of amount of stress on cornea

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DISCUSSION

The biomechanical effect of CXR on cornea increased with low irradiance and high irradiation time (figure 8). With the increase in time cornea becomes rigid at the center and later on gains strength in the periphery. Several mathematical and numerical techniques have been developed to predict the corneal response to external effects. These techniques need representative material constitutive models if they are to produce accurate predictions. However, the material properties of the cornea reported in the literature vary widely, almost within two orders of magnitude, and the variation seems to be caused by a number of primary factors including the effects of the test method, corneal viscoelastic behaviour, the intraocular pressure, and the age.

This is the first experimental study of its type on human corneas to detect the stress on cornea using accelerated protocols. The present study has an edge over the previous few studies based on porcine corneas which used strip extensometry tests which do not simulate in vivo conditions. Further we used laser beams and automated cameras to detect changes in corneal stress using birefringence.

Hafezi et al performed corneal cross-linking study on porcine corneas using strip extensometry technique to determine young's modulus. Their sample size was 50 in each of the four similar groups as ours. Higher light irradiances were associated with lower young's modulus at each percentage strain tested.

Wernli et al studied the effects of cross linking on porcine eyes. Constant irradiation dose with different intensities and illumination times were applied on 10 groups with a control group of 80 eyes. The authors investigated the biomechanical strengthening of ex vivo corneal tissue treated with irradiances between 3 mW/cm2 and 90 mW/cm2 and illumination times from 30 minutes to 1 minute, respectively. Corneal cross-linking in ex vivo tissue show that the Bunsen-Roscoe reciprocity law is only valid for illumination intensities up to approximately 40 to 45 mW/cm2 with illumination times of more than 2 minutes. At higher intensities, the achieved stiffness increase is not significant anymore.

Beshtawi et al used scanning acoustic microscopy to study mechanical properties of eye banked corneas at two different settings of 3 Mw/cm2 and 9Mw/cm2 and found a significant increase in stiffness in these settings as compared to controls. However, they did not see significant difference in these two settings.

This highlights the fact that sample from the same group are similar and the samples of the different group have low cross correlation coefficient, I. e. they are different. This shows that the results of our study are consistent and there is no error in the observations. Of course, the differences are not significant, which is due to the low number of samples.

With 10 min the cornea has gained some strength in the center, that is why we see a dip in the center. Lastly the peripheral side of the cornea also has gained some strength and therefore the stress levels in all the points have come down.

The effect of attached riboflavin to the cornea is seen with the increase in the time period, thus the amount of stress is seen low in the case of 30 min group compared to the 5 min and 30 min groups. This suggests that the cornea gains strength with cross linking which improves with time, ours is the first experiment which has demonstrated that the cornea becomes tougher. Second the protocols suggest that with time the cornea becomes rigid at the center and later on gains strength in the periphery, this could be due to the angle of incidence of light. Variation in birefringence is higher in 5 min and 10 minute group suggesting that the corneas are weak in these two groups.Variation in 30 minutes group is similar to that of the normal cornea, which suggests that a 30 min protocol gives better strength.

CONCLUSION

The amount of stress decreases and the biomechanical strength increases with increase in irradiance time due to longer effect of UV exposure. Cornea becomes rigid in the centre and later on gains strength in the periphery with time, this may be due to angle of incidence of light. Further studies are needed with larger sample size and better protocols to prove the same.

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