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Correlation of Clinical and Biomechanical Outcomes of Accelerated Crosslinking (9 mW/cm^2 in 10 minutes) in Keratoconus with Molecular Expression of Ectasia-Related Genes

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ABSTRACT

Purpose: To assess visual, keratometry, densitometry, and corneal deformation outcomes after accelerated crosslinking (CXL) and its association with gene expression of extracellular matrix proteins.

Methods: 33 eyes underwent accelerated CXL (9 mW/cm^2 for 10 minutes) after epithelium removal. Refraction, visual acuity, keratometry, corneal densitometry, and deformation (Corvis-ST) were assessed before and 6 months after surgery. Epithelium-collected intraoperative was analyzed with qPCR to determine whether the molecular state of disease [lysyl oxidase (LOX), matrix metalloproteinase 9 (MMP 9), transforming growth factor beta (TGFβ), tumor necrosis factor-α (TNFα), interleukin 10 (IL10), interleukin (IL6), collagens (COL IA1 and COL IVA1)] had any bearing on the outcome.

Results: Corrected distance visual acuity (CDVA) remained unchanged ($p > 0.05$). Cylinder ($p = 0.0003$) and spherical equivalent error ($p = 0.02$) reduced significantly after CXL. Keratometry and cone location magnitude index (CLMI) were unchanged after CXL ($p > 0.05$). Corneal densitometry was significantly altered only in the central 0–2 mm region ($p = 0.009$). A new measure of corneal deformation, named corneal stiffness, was also stable after CXL ($p > 0.05$). The preoperative level of different proteins did not influence the clinical outcomes described above ($p > 0.05$).

Conclusion: Accelerated CXL appears to be safe and provides biomechanical stability. Keratometry and refraction remained stable after CXL, with significant improvement in cylindrical error. Molecular expression profile of the keratoconic epithelium did not influence the clinical outcomes.

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Accelerated crosslinking; keratoconus; CLMI; gene expression; biomechanics

Introduction

Accelerated collagen crosslinking (CXL) is a minimally invasive technique to stop or slow down the progression of the disease in corneas with keratoconus.[1] Accelerated CXL enjoys several benefits over the standard method such as less treatment time, less dehydration of the cornea, and less increase in corneal optical haze after the treatment.[2] Kinetic studies on CXL suggest that simply increasing the ultraviolet (UVA) intensity may not result in improved efficacy of the treatment and similar or greater biomechanical strength than conventional CXL.[3] Most accelerated CXL studies have reported similar visual and keratometric outcomes compared to conventional CXL (3 mW/cm^2 for 30 minutes).[4,5]

Corneal epithelial cells and stromal keratocytes produce a number of extracellular matrix proteins and its components lend stability to the corneal ultrastructure. In addition, various stress factors or stimuli can activate the innate inflammatory responses that may have an adverse effect on corneas with keratoconus, which also correlate with the severity grade of keratoconus. UV light may cause an inflammatory response due to the damage caused, which may correspond to the UV energy delivered. A few studies showed that the expression of proteins only transiently altered after conventional CXL in keratocytes after 24 h or patient tears in the long term, suggesting that the treatment might not have influenced the pathological conditions that led to the onset and progression of the disease.[6,7] However, the clinical outcome of accelerated CXL has not been correlated to the pretreatment level of expression yet, e.g., does a cornea with keratoconus with higher level of gene expression result in more haze (densitometry) after CXL? A comprehensive study on visual, keratometry, densitometry, and corneal deformation changes and their correlation with preoperative ocular surface gene expression profiles is still lacking. Therefore, the aim of this study was to assess the changes in all these parameters with the outcomes of accelerated CXL. The incident UV intensity and exposure time was 9 mW/cm^2 and 10 minutes, respectively.

Methods

Study population

The report is a prospective study of clinical characteristics and molecular expression from debrided corneal epithelium of keratoconus subjects who underwent accelerated CXL at the

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Narayana Nethralaya Multi-Specialty Eye Hospital, Bangalore, India. The study was approved by the ethics committee of Narayana Nethralaya Multi-Specialty Hospital (EC approval no. C/2013/03/01). The included subjects met the following criteria before CXL. The age of the patients was restricted between 18 and 60 years. A total of 33 eyes were included with matching preoperative and 6 months postoperative clinical and biomechanics data as well as intra-operative epithelium samples. All patient eyes were graded as per the Amsler–Krumeich classification.[8] Inclusion criteria were mild to moderate keratoconus (grades 1 and 2) with progression defined as an increase in keratometry by at least 1D in 6 months,[9,10] intolerance to contact lens wear, and advanced keratoconus indicated by the thinnest pachymetry lower than 400 μm. Patients with active allergic eye disease, active ocular inflammation, pregnancy, diabetes, glaucoma, or central scarring of the cornea were excluded from the study.

Surgical technique

A single experienced surgeon (RS) performed all the procedures. Topical anesthesia was administered using 0.5% proparacaine hydrochloride (Paracain, Sunways Pvt. Ltd., India). After a lid speculum was placed, central 8 mm of the corneal epithelium was removed with EpiClear™ (Orcasurgical, Kiryat-Shmona, Israel). Then, 0.1% riboflavin in 10% dextran solution (Contacare Ophthalmics and Diagnostics, Vadodra, India) was applied on the exposed cornea surface every 2 minutes for a total of 20 minutes.[11,12] UV-A (wavelength of 365 nm) and incident intensity of 9 mW/cm^2 were applied for the next 10 minutes (Avedro Inc, Waltham, MA, USA), followed by a thorough irrigation with balanced salt solution. This cross-linking procedure delivered a total energy of 5.4 J/cm^2 to the cornea. A bandage contact lens (BCL) was placed for 3 days or until complete healing of the epithelium. The patient was put on a tapering dose of prednisolone acetate 1% (Allergan Inc, Irvine, CA, USA) three times a day for 3 days, two times a day for the next 3 days, and once a day for the next 2 days. Topical antibiotic (Vigamox; Alcon Inc, Fort Worth, TX, USA) was administered three times a day for 1 week. Lubricating eye drop (Systane; Alcon Inc) was administered six to eight times a day for 3 months.

Preoperative and postoperative evaluation

Sphero-cylindrical refractive error and corrected distance visual acuity (CDVA) were assessed before and 6 months after surgery. Keratometry (flat and steep axis), central corneal thickness (CCT), and densitometry on Scheimpflug images were assessed before and 6 months after surgery with Pentacam. Densitometry was assessed in the central 2 mm, 2–6 mm, 6–10 mm, and 10–12 mm annular zone in the anterior (1/3rd of the stromal thickness), middle, and posterior stroma.[13] Axial and tangential cone location magnitude indices (aCLMI and tCLMI) of the anterior surface were also assessed after the treatment.[14] Similarly, corneal deformation was evaluated before and after surgery with Corvis-ST (OCULUS Optikgerate Gmbh, Wetzlar, Germany).

A recent study presented a parallel spring and dashpot model to quantify the corneal stiffness.[15] However, the model did not explicitly delineate the deformation of the extraocular tissues from the total deformation.[15] Recent studies with advanced mathematical models showed that the time scale of corneal deformation during applanation did not allow any significant viscous damping in the cornea.[16] Therefore, a modified spring and dashpot model was introduced, which accounted for not only the extraocular tissue stiffness (Kg) and viscosity (μg) but also an additional spring of stiffness Kc representing the corneal stiffness. Furthermore, the cornea is known to stiffen as the applied stress increases. To model this effect, Kc was assumed to be an exponential function of the applanation pressure, i.e., $Kc = \beta \times e^{\alpha P}$, where P was the applanation pressure. Thus the resultant mathematical equation that describes the corneal and extraocular tissues deformation due to force of the air-puff is as follows:

$$F = Kc(u_1 - u_2) + Kg(u_2) + \mu_g\left(\frac{du_2}{dt}\right)$$

where F is the force, u_1 is the total deformation (reported as deformation amplitude in Corvis-ST), u_2 is the deformation of the extraocular tissue, and t is the instantaneous time during applanation. Using this formulation, it is possible to accurately describe the corneal, extraocular tissue, and total deformation. The above equation was solved using a combination of least squares and finite difference technique to determine the magnitude of β, α, Kg, and μg before and 6 months after CXL. The applanation pressure in the device ranged from 0 to 180 mmHg. For each analyzed waveform, an aggregate stiffness $\left(\widetilde{Kc}\right)$ was defined and was equal to the mean of Kc's at all applanation pressures. $\widetilde{Kc}$, Kg, and μg were used for statistical comparison.

Additional corneal deformation variables analyzed were area under the corneal deformation curve, area under the extraocular tissue deformation curve, area under the deformation amplitude curve, deformation amplitude, time of first applanation and second applanation, time of highest concavity, and deformation amplitude at time of first applanation and second applanation.

Assessment of gene expression in corneal epithelium

The corneal epithelium was debrided during the CXL procedure. Debrided epithelial cells were immediately transferred to −80°C for storage till processing for RNA extraction. Extraction of mRNA and analysis of gene expression were carried out as described before.[17,18] Briefly, TRIZOL reagent was used for total RNA extraction as per the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA), which was then quantified and quality assessed. cDNA synthesis was performed using an iScript cDNA conversion kit (Bio-Rad, Philadelphia, PA, USA), followed by real-time PCR using a CFX Connect™ real-time PCR detection system (Bio-Rad). Total levels of lysyl oxidase (LOX), matrix metalloproteinase 9 (MMP 9), transforming growth factor beta (TGFβ), tumor necrosis factor-α (TNFα), interleukin 10 (IL10), interleukin (IL6),

and collagens (COL IA1 and COL IVA1) were estimated after normalization to actin.

Statistical analyses

Normality of distribution was assessed for all distributions. If variables were normally distributed, paired t-test was used to compare preoperative and postoperative values. If variables were non-normally distributed, paired Wilcoxon test was used. Accordingly, correlation was assessed either with Pearson or with Spearman correlation coefficient. Mean values were reported as mean ± standard of the mean. Median values were reported with their corresponding 95% confidence interval (CI).

Results

Clinical characteristics of the patient study cohort are detailed in Table 1 and Table 2. Median CDVA, spherical error, and spherical equivalent remained unchanged after surgery ($p > 0.05$). Median cylindrical error reduced significantly after surgery ($p = 0.0003$). Median flat and steep axis keratometry remained unchanged as well after surgery ($p > 0.05$). However, a significant decrease in CCT was noted after surgery ($p = 0.0001$). The mean values of anterior and posterior cone location magnitude index (CLMI) based on axial or tangential curvatures were determined. aCLMI decreased significantly after treatment ($p = 0.003$) (Table 2). However, tCLMI remained unchanged after treatment ($p = 0.05$) (Table 2). No change in densitometry was found in either the central or posterior cornea in all the zones ($p > 0.05$). However, the central 0–2 mm anterior cornea showed significant increase in densitometry at follow-up (13.22 ± 0.36 vs. 15.84 ± 0.87, $p = 0.009$). The remaining zones (2–6 mm, 6–10 mm, and 10–12 mm) of the anterior cornea did not show any significant increase in densitometry ($p = 0.1$, 0.1, and 0.07, respectively). None of the biomarkers correlated with the change in cylinder ($p > 0.05$), change in densitometry of the central 0–2 mm anterior cornea ($p > 0.05$), and change in CCT ($p > 0.05$).

Mean α was $4.48 \times 10^{-3} \pm 6.7 \times 10^{-4}$ $mmHg^{-1}$ and $4.35 \times 10^{-3} \pm 7.8 \times 10^{-4}$ $mmHg^{-1}$ before and after surgery, respectively ($p = 0.89$). Mean β was 66.17 ± 5.19 N/m and 71.62 ± 6.93 N/m before and after surgery, respectively ($p = 0.47$). Mean $\widetilde{Kc}$ (96.49 ± 2.77 N/m vs. 99.48 ± 4.38 N/m, $p = 0.46$), Kg (350.67 ± 33.08 N/m vs. 317.07 ± 30.01 N/m, $p = 0.23$) and μg (2.15 ± 0.13 $N.sec/m^2$ vs. 2.15 ± 0.14 $N.sec/m^2$, $p = 0.99$) were unchanged after the surgery. $\widetilde{Kc}$ was positively correlated with CCT (r = +0.58, $p = 0.01$) but not with IOP ($p = 0.47$). Similarly no change in area under the corneal deformation curve ($p = 0.73$), area under the extraocular tissue deformation curve ($p = 0.19$), and area under the deformation amplitude curve ($p = 0.10$) was detected after surgery. Deformation amplitude increased but did not achieve significance (1.19 ± 0.018 mm vs. 1.22 ± 0.018 mm, $p = 0.08$). Furthermore, times of first applanation (7.17 ± .06 sec vs. 7.26 ± 0.04 sec, $p = 0.14$), second applanation (21.58 ± 0.07 vs. 21.49 ± 0.05 sec, $p = 0.18$) and highest concavity (15.03 ± 0.11 sec vs. 15.06 ± 0.09 sec, $p = 0.85$) were unchanged after surgery. The only corneal deformation variable that underwent significant change after surgery was deformation amplitude at the time of second applanation (0.35 ± 0.01 mm vs. 0.38 ± 0.01 mm, $p = 0.004$).

Since deformation amplitude at the time of second applanation was the only deformation variable that achieved significance, Spearman correlation coefficient was assessed between change in deformation amplitude at the time of second applanation and expression of the biomarkers. We chose the pro-inflammatory cytokine IL6 and its antagonist cytokine IL10, collagen isoforms IA1 and IVA1, collagen crosslinking enzyme LOX, extracellular matrix degrading enzyme MMP9, and its inhibitor TIMP1 for gene expression analysis based on our previously published data regarding deregulation of these genes (Table 3).[17,18] None of the preoperative biomarker gene expression levels (IL10: $p = 0.43$, IL6: $p = 0.89$, LOX: $p = 0.61$; MMP9: $p = 0.69$; Col IA1: $p = 0.71$; Col IVA1: $p = 0.69$; TIMP1; $p = 0.81$) showed any significant correlation with the change in deformation amplitude at the time of second applanation.

Discussion

Accelerated CXL has the advantage of reduced treatment time and can increase the patient flow in the clinic. Both conventional and accelerated CXL are safe procedures and temporary haze after CXL is mostly due to temporary changes in the hydration level of the tissue with no damage to the endothelium.[2] The short-term (up to 6 months) refractive and keratometric outcomes were similar between conventional and accelerated CXL. Analyses of the corneal deformation using ocular response analyzer (ORA, Reichert Inc., Depew, NY, USA) yielded similar results between

Table 1. Clinical characteristics of the study cohort.

	Before Surgery		Month 6		
	Median	95% CI	Median	95% CI	*p*-value
Spherical Error (D)	−1.38	−2.50 to 0.00	−1.0	−2.50 to 0.00	0.86
Cylindrical Error (D)	−3.5	−4.09 to −2.50	−2.25	−3.00 to −2.00	0.0003
Spherical Equivalent	−3.63	−5.30 to −1.75	−2.75	−4.25 to −1.13	0.02
CDVA (LogMAR)	0.1	0.00 to 0.20	0.1	0.00 to 0.10	0.56
K1 (D)	45.1	43.27 to 46.40	44.8	42.92 to 46.38	0.62
K2 (D)	48.7	47.45 to 50.87	48.7	47.00 to 50.98	0.52
Kmax (D)	52.6	50.68 to 55.40	52.3	50.28 to 55.24	0.56
CCT (μm)	469	454.65 to 480.05	461	443.41 to 471.77	0.0001

Note: Median [with 95% confidence interval (CI)] refractive and tomographic outcome of the eyes before and after crosslinking (n = 21).

Table 2. Preoperative and postoperative cone location magnitude indices.

	Before Surgery	Month 6	
	Mean	Mean	*p*-value
Anterior axial aCLMI (D)	5.64 ± 0.53	5.18 ± 0.49	0.003
Anterior Tangential tCLMI (D)	9.15 ± 0.65	8.66 ± 0.68	0.05

Note: Mean ± standard error of the mean of the anterior and posterior cone location magnitude index (CLMI) based on axial and tangential curvature (n = 21).

Table 3. Expression levels of ectasia-related genes.

	Mean	SEM
Col IA1	4.75	4.19
Col IVA1	1.21	0.99
IL-10	0.35	0.26
IL-6	2.12	1.12
LOX	0.49	0.36
MMP-9	1.53	1.32
TIMP1	0.21	0.14

Note: Average normalized fold change in the expression levels of the indicated genes determined in patient corneal epithelium collected during the cross-linking procedure (mean± standard error of the mean) within the study cohort (n = 21). All threshold cycle data are normalized to the corresponding actin gene expression (dCt).

conventional and accelerated CXL.[21] However, the results from the ORA should be treated with caution as ORA is known to be insensitive to structural changes in the corneas after CXL[22] and may not be considered as a suitable quantifier of corneal biomechanical changes. Another study used the same UV intensity and irradiation time as the present study and found no statistically significant change in CDVA or sphero-cylindrical refractive error at 1 year after treatment.[23] Other variables such as steep and flat axis keratometry also remained stable after treatment, similar to the present study.[21] However, a recent study on accelerated CXL using 30 mW/cm^2 for 3 minutes observed not only an improvement in uncorrected distance visual acuity but also a significant reduction in mean maximum keratometry by ~0.8D.[24]

In this study, CDVA was unaffected after CXL. Cylinder and spherical equivalent refractive errors were reduced significantly, indicating some corneal flattening. However, this flattening effect was not significant among the CLMI indices. Flattening of the cornea is expected to occur at the stroma–epithelium interface and, therefore, the epithelium may mask some of the flattening of the cornea after CXL. Regional densitometry was significantly higher only in the anterior central cornea. While CLMI has not been assessed after CXL, densitometry tends to increase after CXL, which has been shown previously.[13] Corneal deformation using Corvis-ST was also assessed and was found to be relatively unchanged after accelerated CXL. Mean corneal stiffness ($\widetilde{Kc}$) was stable after CXL, indicating stabilization of the disease. This is the first study where quantitative data on the change in corneal stiffness *in vivo* after accelerated CXL has been described. The equation used to derive corneal stiffness is a refined version of the proposed model in an earlier study since it was able to incorporate explicit assessment of both corneal and extraocular tissue stiffness.[17,10] Since corneal stiffness was significantly correlated with CCT, it indicated that keratoconic corneas may become biomechanically weaker with increasing severity of keratoconus. The lack of significant topographic flattening may be attributed to the following reasons: (a) stiffening effect of accelerated CXL is significantly lower than conventional 30 minute treatment[25]; (b) most treated corneas in this study were in the early stage of the disease and topographic flattening is expected to be more in the mild form of keratoconus.[26] In this study, none of the eyes regressed after the treatment, indicating that accelerated CXL stabilized the biomechanical condition of the cornea but wasn't able to provide significant topographic flattening.

The molecular expression profile of the disease correlates with the severity of keratoconus,[17,18] suggesting the predictive utility of molecular factors. Hence we selected a set of molecular markers that have already been proven to have differential gene expression patterns in keratoconus corneal epithelium when compared with controls.[17,18] Furthermore, gene expression levels of certain factors such as LOX, MMP9, and collagens in the patient epithelium also correlated with grades of keratoconus; however, significant correlation was observed typically in the higher grades.[17,18] Therefore, within a cohort of Grade 1–2 keratoconus patients, we attempted to evaluate the correlation of preoperative gene expression levels with the outcome measures of CXL. The genes that were selected are known to be involved in keratoconus pathology and in corneal structure.[19,20,27–29] The aim of these experiments was to assess whether gross differences in preoperative corneal gene expression resulted in less than optimal clinical outcomes after accelerated CXL. A recent study using tears from keratoconus patients assessed before and after CXL did find some differences in the tear expression levels of cytokines at 6 and 12 months after CXL,[6] but the trends were inconsistent over a year of observation. The study did report changes in the first 2–3 weeks,[6] but the expression level was influenced by the use of topical steroids and hence the significance of observed differences may be skewed. In addition, tear levels may be influenced by systemic factors as well as environmental ones. Hence analyzing local gene expression in the corneal epithelium may prove to be a more robust predictor of molecular changes. However, our gene expression results of the epithelium did not correlate with clinical or corneal deformation. One of the reasons may be that within the cohort, the outcomes of the actual procedure were not significantly heterogeneous, and hence the lack of correlation with predictive biomarkers, suggesting that a larger cohort might be necessary for future studies. However, we have previously observed in patients that the biomarker levels do not significantly change within the early grades of keratoconus in large cohort studies.[17,18] Thus, the data suggests that gene expression levels of these selected markers in the corneal epithelium may not have a significant bearing on the outcome of collagen CXL. Alternatively, there may be other undiscovered biomarkers that may be more indicative of the outcomes of CXL.

Conclusion

Our data indicate that the accelerated CXL procedure might not have significantly measurable effects on the corneal strength of treated keratoconus patients within the 6-month follow-up time frame. Additionally in the study cohort, we did

not observe any association of the epithelial gene expression levels with the outcomes of accelerated corneal collagen CXL.

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Declaration of interest

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