

In Vitro Effect of the Biomechanics After Photodynamic Therapy (PDT) Intervention on Rabbit Cornea

Guanyu Su

Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University

Shaofeng Han

Beihang University

Bowen Song

Wayne State University School of Medicine

Kai Cao

Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University

Qingfeng Liang (✉ lqflucky@163.com)

Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University

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Abstract

Objective: To observe the change of the biomechanical properties of rabbit cornea after the intervention by corneal collagen cross linking (CXL) and toluidine blue O combined with red light (TBOR).

Methods: The study was carried out in compliance with the ARRIVE guidelines. 20 healthy adult New Zealand white rabbits were randomly divided into two groups. One group was taken with Riboflavin combined with UV light therapy in the right eye of healthy New Zealand white rabbits, and the other group was taken toluidine blue O combined with red light (TBOR) treatment. All left eyes were taken as controls. Parameters, from Pentacam and Corvis ST like K1 (The keratometry readings of the flattest), K2 (The keratometry readings of the steepest), ACD (Anterior chamber depth), Pupil diameter, ACA (Mean angle of the anterior chamber), ACV (Volume of the anterior chamber), bIOP (Biomechanical Intraocular pressure), CT (Corneal thickness, pachymetry), A1 Time (Time from starting until the first applanation), A1 Velocity (Corneal speed during the first applanation moment), A2 Time (Time from starting until the second applanation), A2 Velocity (Corneal speed during the second applanation moment), HC Time (Time from starting until highest concavity is reached), Peak Dist (Distance of the two “peaks” at highest concavity) and HC-Radius (The radius at highest concavity), were examined before and 2 weeks after intervention. The rabbits were sacrificed after anesthesia. Then, their corneas were removed for corneal stretch test.

Results: With the examination of Pentacam and Corvis ST, IOP (11.28 ± 11.2 mmHg v.s. 6.66 ± 4.02 mmHg) and A1 time (7.03 ± 1.27 s v.s. 6.55 ± 0.35 s) were increased, comparing with those before intervention. From the in vitro corneal stretch test, the tangent modulus of the CXL group was more than 3 times of the Control group, whereas the tangent modulus of the TBOR group was about 0.7 times to that of the Control group.

Conclusions: From the rabbit cornea intervention with CXL and TBOR, CXL showed an obvious effect of increasing the hardness of rabbit cornea, while TBOR may did no help to increase the hardness of cornea.

Introduction

With unique structure^{1, 2} and biomechanical properties,^{3 4} cornea can maintain the transparency and provide a clear vision. In recent years, corneal collagen cross linking (CXL) was applied in the field of the treatment of keratoconus and infectious corneal disease. This technique can not only kill bacteria but also can increase corneal rigidity and the resistance of degradation ability of enzyme.⁵ In vitro experiments conducted by Martins et al.⁶ demonstrated corneas intervened by riboflavin combined with UVA (CXL) could inhibit the growth of bacterial drug-resistance. Tissues targeted by irradiation of special light causes transformation of oxygen into ROS (reactive oxygen species) in the aerobic environment around the excited state energy, this mechanism can be applied many different organelles producing toxic effect which causes cell death.⁷ Different light sources, such as Ultraviolet (400 nm),⁸ blue light (440 ~ 480 nm),⁹ green light (500 ~ 560 nm)¹⁰ and red light (600 ~ 780 nm)¹¹ combined with different

photosensitizers such as riboflavin,¹² Rose Bengal,¹⁰ 5-aminolevulinic acid,¹³ Chlorin e6¹⁴ and toluidine blue¹¹ can create different types of PDT. In previous study, our group had confirmed that CXL¹⁵ and TBOR¹⁶ had an apparent inhibitory effect on bacterial growth through in vitro studies. In this study, we will investigate the effects of PDTs on biomechanics properties of corneas by biomechanical examination following PDT treatments.

Materials And Methods

Experimental animals

The study was carried out in compliance with the ARRIVE guidelines. All operations involving animals were carried out in accordance with the standard established by the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Our study protocol was approved by the Animal Care and Use Committee, Capital Medical University (AEEI-2017-081). Twenty healthy adult New Zealand white rabbits (both genders, aged 3–4 months, weight 2.5–3.0 kg) were provided by the Beijing Longan Research Animal Center (License NO. SCXK Beijing 2-14- 0003). The right eye of each animal was selected as the experiment group, whereas, left eye was taken as the a control group.

Experimental design

Twenty New Zealand white rabbits were randomly divided into two groups. Ten in each of the CXL and TBOR groups. Riboflavin (0.1wt%) combined with UV therapy (5.273mw /cm²,30min) and toluidine blue O (1mmol/L) combined with red light (87.8mw /cm²,30min) were applied to intervene in the right eye grouping of New Zealand white rabbits individually (Figure 1).

In vivo biomechanical examination

The corneas of each group were examined by Pentacam (Oculus, Wetzlar, Germany) and Corvis ST (Oculus, Wetzlar, Germany) at different time points (before intervention and 2 weeks after intervention). During the examination, two assistants were required. One assistant wrapped the surgical towel around the experimental rabbit with only the head exposed. The experimental eyes were placed in front of the test lens following the adjustment of its position and direction until a clear visual appears on the monitor. Interventions will then be carried out. Detailed parameters are shown in Table 1 and Table 2.

In vitro biomechanical testing

Two weeks after the interventions, rabbits were euthanized with an intravenous overdose injection of 5% pentobarbital (1 mg/kg). Both eyes of each rabbit were immediately enucleated. The corresponding 20 left eyes constituted the control group. Cornea is removed using an ophthalmic scalpel and interstitial scissors along the periphery of the rabbit cornea about 2-3mm away from the sclera at room temperature. Other ocular components were also removed. Corneal strips of about 3mm width were made with a two-

parallel surgical blades¹⁹(Figure 2A) in the inferior-superior direction. (Note that the corneal strips should have 2-3mm sclera at both ends)

The strips were connected to a pair of mechanical clamps, leaving 10mm between the two clamps. Then material testing machine (EZ Test Compact Table-Top Universal Tester, Shimadzu, Kyoto, Japan) was used for uniaxial tension test on the corneas (Figure 2B). The sclera part at both ends of the cornea strip was clamped on both ends. The machine was equipped with a 50 N capacity load cell, the test was conducted at room temperature. The humidity around the test was reduced by air humidifier to simulate the tissue environment. The initial distance between the two clamps with a Vernier caliper was measured and recorded it as L_0 . We carried out four loading and unloading cycles on samples with the 1mm /min tensile rate and 0.10N as the maximum load (Figure 2C). The behavior recorded in the fourth cycle was considered to be representative of the sample stability results.²⁰

The load-displacement ($F - \Delta L$) data for the fourth cycle was used to calculate the stress under each load(F), stress is $\sigma = F/wt$ (t was the average corneal thickness and w is the average specimen width). The related strain was obtained as $\epsilon = \Delta L/L_0$. The result of stress-strain conforms to the fitting function-exponential function $\sigma = A(e^{B\epsilon} - 1)$, where A and B are constants. The tangent modulus E_t is calculated as $E_t = d\sigma/d\epsilon = A B e^{B\epsilon} = B(\sigma + A)$ ¹⁹.

Statistical analysis

All analysis was performed using the SPSS 24.0 (SPSS Inc., Chicago, USA). Comparisons between biomechanical parameters in the two different groups were performed using the paired T-test. P values ≤ 0.05 were considered statistically significant.

Results

Biomechanical behavior

After intervention (CXL and TBOR), The bIOP (11.28 ± 11.2 mmHg) was greater than before intervention (6.66 ± 4.02 mmHg), but no significant difference ($s = 35.50$, $P = 0.07$). Furthermore, A1 time (7.03 ± 1.27 s) increased, compared with before intervention (6.55 ± 0.35 s), however, there were no significant difference ($t = 34$, $P = 0.08$). There was no statistically significant difference about other relevant biomechanical indicators comparing before intervention: Pachymetry ($S = 4.50$, $P = 0.33$), A2 time ($t = 1.32$, $P = 0.13$), A1 Velocity ($t = 1.01$, $P = 0.13$), A2 Velocity ($t = 0.40$, $P = 0.69$), HC Time ($t = 1.10$, $P = 0.29$), Peak Dist ($S = 6.00$, $P = 0.76$), and HC-Radius ($t = 0.96$, $P = 0.35$) (Appendix Table 1). According to the Pentacam test results of the anterior segment analysis measurement system, there was no statistical difference in K1, K2, ACD, Pupil diameter, CA Mean and C. Volume after photodynamic intervention (CXL and TBOR) (Appendix Table 2).

Uniaxial tension test

As shown in Figure 3, the curves of load-displacement diagrams of the three groups are obviously different, which was determined by parameters A and B (Table 3). Through the stress-strain (σ - ϵ) relationships (Figure 4), the tangent modulus ($E_t = d\sigma/d\epsilon$) can be obtained at any stress level. The tangent modulus (E_t values) of the three groups (10, 20 and 30kPa stresses) were varied significantly (Table 4). The E_t value of CXL was significantly higher than other groups at the same stress level. For all 3 stress levels, the difference of E_t value in TBOR group (0.684 ± 0.064 , 0.748 ± 0.100 and 0.813 ± 0.135 MPa), when compared to CXL group (3.261 ± 0.230 , 3.570 ± 0.355 and 3.879 ± 0.481 MPa) and control group (0.989 ± 0.078 , 1.054 ± 0.120 and 1.119 ± 0.162 MPa), was minimal. However, the E_t value of the CXL group was more than 3 times (284.1~383.2%, 273.9~420.2%, 265.3~455.6%) higher than that of the control group, and the E_t value of the TBOR group was only 0.7 times (58.1~82.1%, 55.2~90.8%, 52.9~99.1%) to that of the Control group.

Discussion

Our study attempted to address the biomechanics of cornea after different PDT treatments. Results demonstrated that CXL intervention resulted in significantly increase in the mechanical stiffness (as measured by tangent modulus, E_t) of the ex-vivo rabbits' cornea. However, the effect of TBOR method on corneal hardness was not established. The changes in corneal biomechanics in vivo were not significant.

Little attention has been given to the effect of PDT on cornea biomechanics. Previous studies on the biomechanical properties of cornea were largely CXL clinical studies using Pentacam,²¹⁻²³ Corvis ST^{23,24} and ORA (Ocular response analyzer)^{25,26}. Nevertheless, the biomechanics metrics provided by Pentacam and Corvis ST cannot be linked directly to the traditional measures of tissue stiffness (here is mainly E_t). At present, most of the PDT studies on cornea focus on CXL technique. It showed that CXL can increase the corneal hardness and prevent the development of keratoconus.^{21,27} CXL has also been shown to increase the corneal hardness in a number of animal studies.^{28,29} In addition, current studies only used traditional Young's modulus to study changes in the biomechanical properties of the cornea in vivo.²⁷

Tangent modulus (E_t) refers to the ratio of instantaneous stress and instantaneous strain of a tissue material at a some timepoint.^{30,31} E_t value is rarely used as to evaluate the change of biomechanical property in cornea. While there is no agreement on the effect of cornea's rigidness after CXL. With E_t values analysis method, we found that CXL could effectively increase the hardness of rabbit cornea. This conclusion was also consistent with the study of Wollensak et al.²⁸ and others.^{27,29,31,32} Our previous studies demonstrated the effectiveness of TBOR on the treatment of bacterial keratitis.^{11,16} However, few researchers have studied the effect of TBOR on corneal biomechanics, or comparing the E_t value of CXL and TBOR on corneal biomechanics.

In this study, corneal hardness was significantly increased after CXL intervention, while TBOR had little effect on corneal hardness. TBOR's E_t values were lower to that of the control group. According to the optical knowledge, red light wavelength is about 630 nm, UV wavelength is about 370 nm, $c = \lambda * f$ (c is the speed of light, λ is wavelength, f is frequency), the longer the wavelength, the lower the frequency.

According to the formula “ $E = h * f$ (E is energy, h is Planck's constant)”, the higher the frequency, the higher energy and the stronger penetrating power. Therefore, the higher UV energy has stronger penetrating power. Based on these facts, we believe that UV light can easily penetrate epithelial layer of the cornea, reach the stroma layer and induce the matrix layer fiber to arrange more closely, thus harden the cornea.

Our studies demonstrated the change of the biomechanical indexes of rabbit corneas by two kinds of PDT methods (both CXL and TBOR) before and after the intervention. Results show that after the intervention of the two methods, Et value of CXL group increased significantly compared to the TBOR group and the control group. Using animal model, we verified the clinical effectiveness of using CXL in slowing the progressive of keratoconus^{21, 22, 32}. However, TBOR therapy did not increase the biomechanical properties of the cornea. Future investigators should be aware that TBOR may not affect the biomechanical properties of the cornea.

This study has some limitations. Firstly, our sample size was limited to only 10 rabbits in each group. So larger sample size maybe necessary to further study the effect of CXL and TBOR on cornea biomechanics. Secondly, our tests were done ex vivo, we tried to preserve the cornea tissue and examine them within 2 hours post-mortem, degradations of the cornea might still occur, which might lead to inaccurate results. Thus, further tests should be done with the limitations in mind.

Overall, we found that CXL can effectively increase the biomechanical properties of cornea and significantly improve corneal hardness. But toluidine blue O combined with red light (TBOR) therapy did not result in significant increase in corneal hardness.

Declarations

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Competing interests: The authors declare that there is no conflict of interest.

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Corresponding author: Prof. Qingfeng Liang, MD, PhD, Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Key Laboratory of Ophthalmology and Visual Sciences, Beijing, 100005, China.

E-mail : lqflucky@163.com ; Phone: +86-10-58265938; Fax: +86-10-65125617.

Ethics approval and consent to participate All procedures involving animals were carried out in accordance with standards established by the Association for the Research in Vision and Ophthalmology. Our study protocol was approved by the Animal Care and Use Committee, Capital Medical University

(AEEI-2017-081). The protocol adhered to the policy and guidelines regarding the care and use of research animals.

Consent for publication This study was published with the consent of all authors

Availability of data and materials The datasets used during the current study are available from the corresponding author and first author on reasonable request.

Authors' Contributions: Qingfeng Liang directed the whole process of the study and sponsored the study; Guanyu Su was the main author of the study and wrote the paper; Shaofeng Han was the author of Figure 3, Figure 4, Table 3 and Table 4 of the study; Bowen Song polished the language of the paper; and Kai Cao was the statistical analysis of the study.

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Tables

Table 1 Parameters derived from Pentacam and their meanings

Parameters	Means\unit\
K1	The keratometry readings of the flattest\D\
K2	The keratometry readings of the steepest\D\
ACD	The distance from the corneal epithelia to the lens, The anterior chamber depth \mm\
Pupil diameter	The Diameter of pupil\mm\
C.A.Mean	Mean angle of the anterior chamber\°\
C.Volume	The volume of the anterior chamber\mm ³ \

Table 2 Parameters derived from Corvis ST and their meanings

Parameters	Means\unit\
biOP	Biomechanical Intraocular pressure\mmHg\
Pachymetry	The corneal thickness\um\
A1 Time	Time from starting until the first applanation \ms\
A1 Velocity	Corneal speed during the first applanation moment\msec\
A2 Time	Time from starting until the second applanation\ms\
A2 Velocity	Corneal speed during the second applanation moment\msec\
HC Time	Time from starting until highest concavity is reached\ms\
Peak Dist	Distance of the two “peaks” at highest concavity (HC) \mm\
HC-Radius	The radius at highest concavity (HC) \mm\

Table 3 Mean and standard deviation of constitutive parameters A and B in the three specimen groups

Group	A	B	RMS, Mpa
CXL Group	0.096±0.008	30.894±12.555	0.00404±0.000901
TBOR Group	0.097±0.008	6.419±3.549	0.00268±0.000193
Control Group	0.142±0.009	6.494±4.216	0.00556±0.003

Table 4. Average and standard deviation values of tangent modulus (MPa) in treated and control groups at three stress levels.

Stress(Kpa)	Tangent Modulus, Et (MPa)				
	CXL	TBOR	Control	Et _{CX} /Et _{Co} , %	Et _{TB} /Et _{Co} , %
10	3.261±0.230	0.684±0.064	0.989±0.078	284.1~383.2	58.1~82.1
20	3.570±0.355	0.748±0.100	1.054±0.120	273.9~420.2	55.2~90.8
30	3.879±0.481	0.813±0.135	1.119±0.162	265.3~455.6	52.9~99.1

Note Et_{CX}/Et_{Co} = ratio between tangent modulus in CXL group (Et_{CX}) and Control group (Et_{Co}), Et_{TB}/Et_{Co} = ratio between tangent modulus in TBOR group (Et_{TB}) and Control group (Et_{Co})

Figures

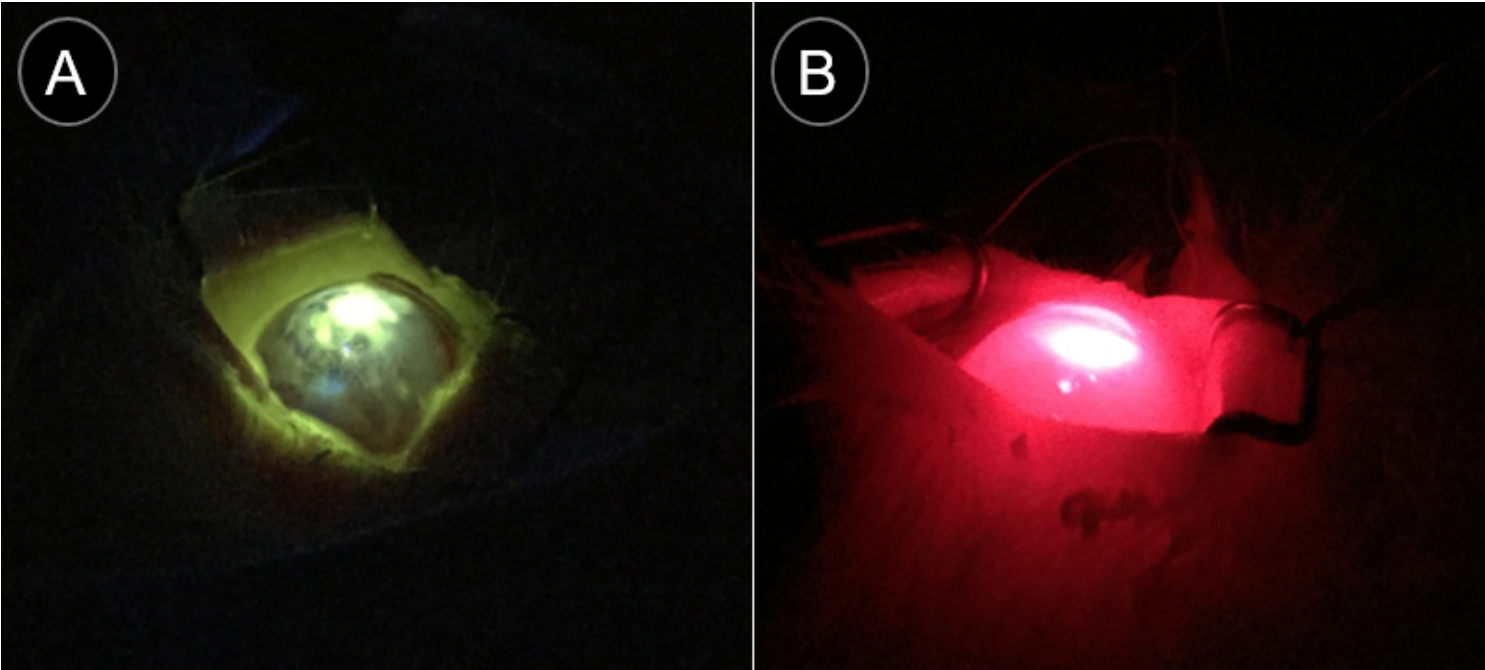


Figure 1

Corneas in the treatment of CXL and TBOR

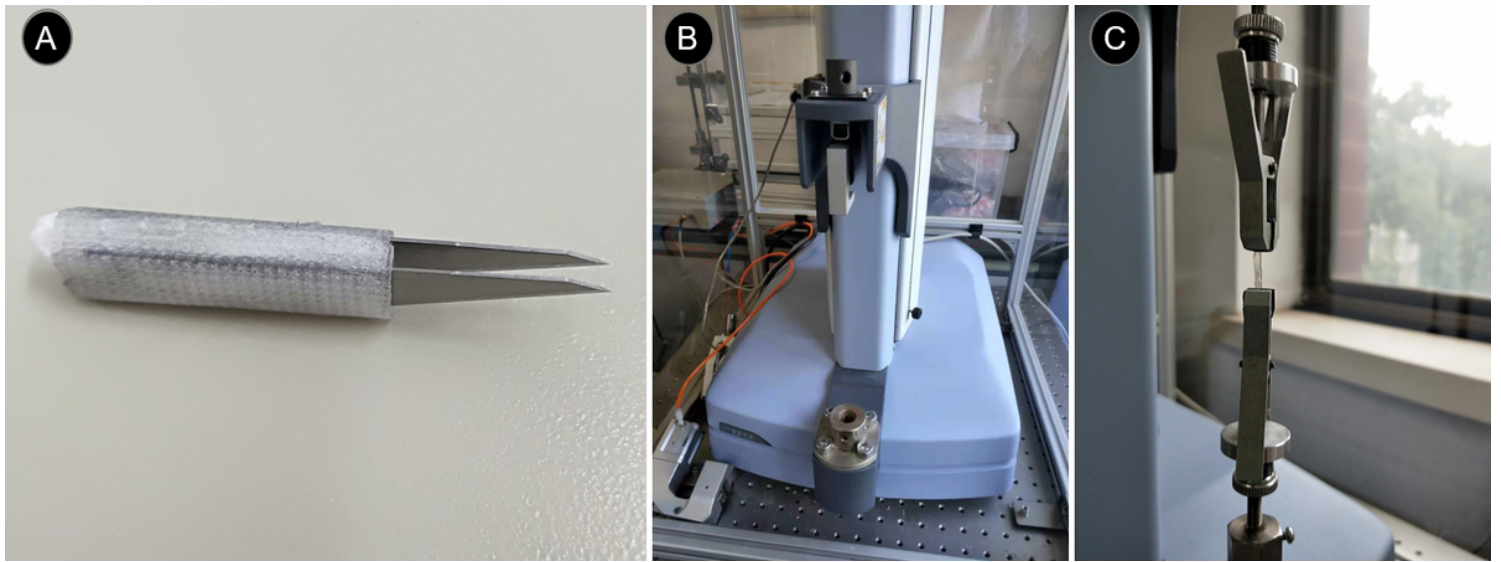


Figure 2

A. Two parallel surgical blades B. material testing machine C. Corneal strips and stretching platforms

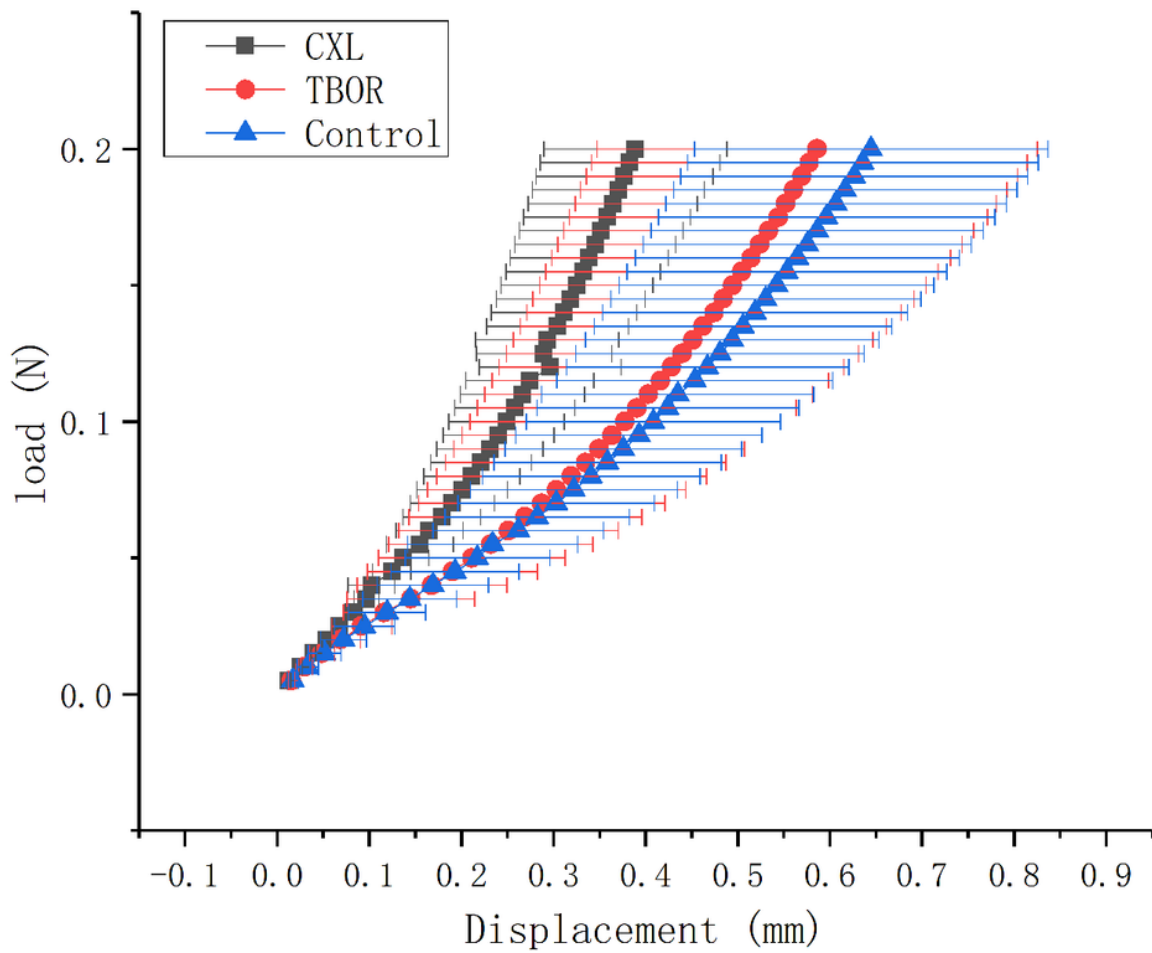


Figure 3

Mean load-displacement behavior in three groups.

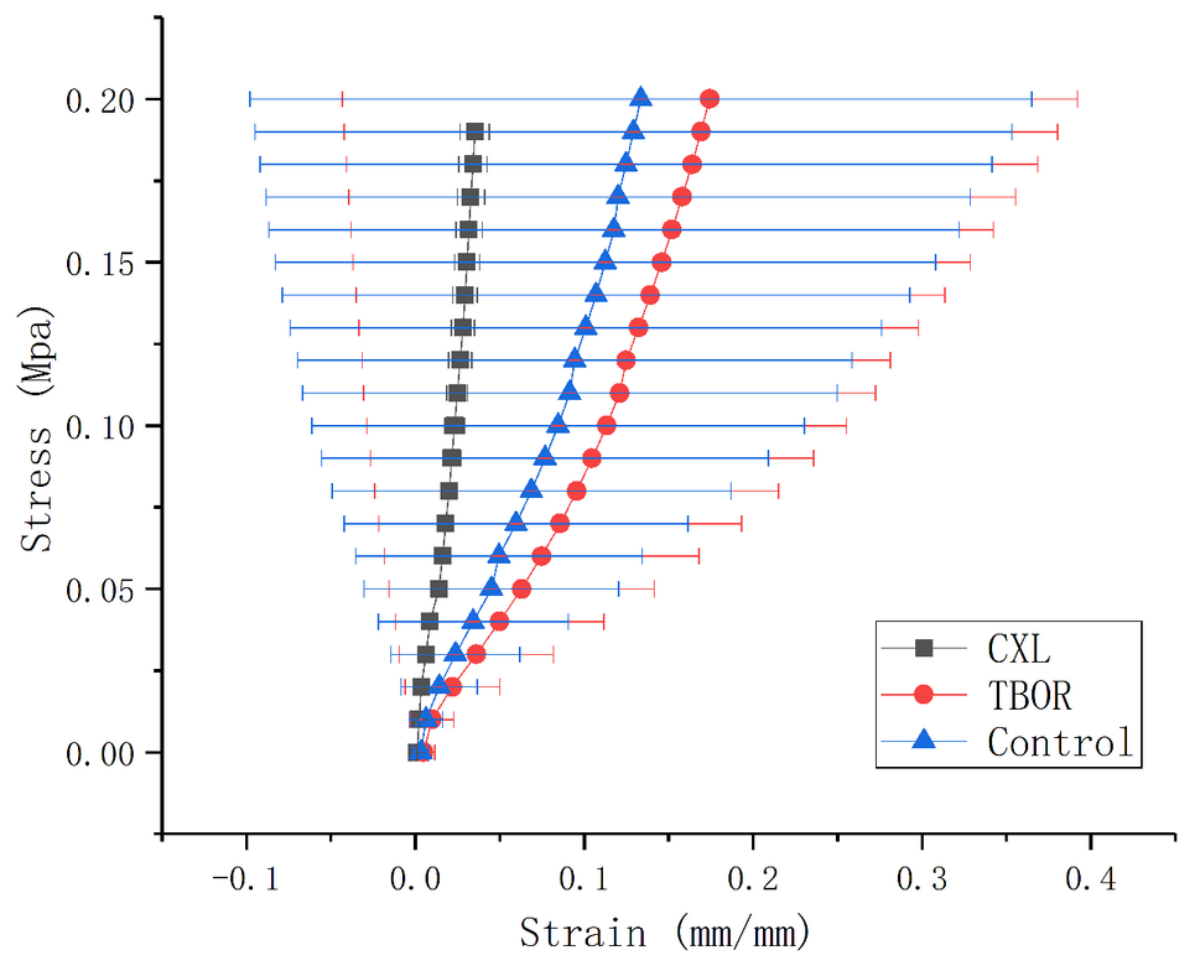


Figure 4

Mean corneal stress-strain behavior of corneas in three groups, error bars represent the standard deviation of strain values.

Supplementary Files

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