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# Biomechanical and morphological changes of rabbit corneas under collagenase type II and negative pressure: three months follow-up observation

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## *Abstract*

***Background:*** To investigate biomechanical and morphological changes of rabbit cornea ectasia induced by collagenase type II and negative pressure during 3 months after treatment.

***Method*:** Eighteen New Zealand white rabbits were randomly and evenly arranged into three groups. The left corneas were continuously treated by negative pressure suction (NP group) with 500 mmHg for 30 min once two days, three times in total. The central area of left corneas were soaked in the collagenase type II (CII group) solution (200 μL of 3 mg/ml) for 30 min. The left corneas (CP group) were disposed as CII group firstly, then applied negative pressure suction as NP group for once after 5 days. All right corneas were treated as control eyes. Corneal morphology parameters and Ocular Response Analyzer (ORA) output parameters were collected in vivo once

a week for three weeks after treatment and before execution. Histology and biomechanics were tested in vitro at the third month after treatment. Paired t-test and repeated measures analysis were used to determine if there were differences in biomechanical and morphological related parameters across time.

***Results*:** In NP group, corneal thickness and diopter changed to some extent after treatment immediately, and the elastic modulus increased and relaxation degree slowed after 3 months. In CII group, corneal diopter increased, corneal central thickness (CCT) and corneal hysteresis (CH) decreased at the second week after treatment, which showed the characters of ectatic corneas. Then the degree of ectasia decreased with time. No regular changes was found on experimental corneas in CP group.

***Conclusions*:** Collagenase type II results in ectatic corneas around two weeks after treatment, but the degree of ectasia decreased with time, and there was no significant difference compared with the controls after 3 months. After negative pressure suction, corneal morphology changed in a short period, and elastic modulus increased and relaxation time increased after a three months recovery, indicating that the negative pressure suction do have a certain effect on corneas.

***Keywords:*** biomechanical property; negative pressure; collagenase; ectatic corneas

# Background

The cornea is the transparent front part of the eyeball, and plays an important role in the refractive system of the eye. Corneal refractive index is closely related to the corneal morphology, which is determined by intraocular pressure, corneal biomechanical properties and so on [1]. Corneal ectasia is a disease of refractive instability with progressive destruction of corneal structure [2], characterized by central and paracentral corneal stroma thinning, corneal protrusion, irregular astigmatism and myopia [3, 4], including keratoconus and post-refractive surgery ectasia [5, 6]. For now, the pathogenesis of corneal ectasia remains ill defined, further researches are needed.

An animal model with corneal anatomy and physiology similar to human being is a valuable and indispensable tool in basic researches. Genetic approach, corneal stromal ablation, and enzyme have been applied to construct corneal ectasia animal models [7-15]. Tachibana et al. [8] constructed spontaneous mutated mice with keratoconus appearance, which are more suitable for genetic and molecular researches. The post-laser in situ keratomileusis corneal ectasia model is constructed by cutting the corneal stroma [9, 10], while not commonly used due to the high modeling cost. Enzyme-mediated corneal ectasia models have been tried [11-15] by chondroitinase ABC or collagenase digestion, while they were mostly based on corneas in vitro, or only made short-term observations (like 14 days) in vivo. Enzyme can lead to fibrinolysis and a series of remodeling processes of corneal stroma. During corneal stromal remodeling, the corneal morphology and mechanical properties, which are

two important factors leading to the cornea ectasia [16, 17], may be in an unstable state. If a treatment of corneal ectasia (like corneal collagen crosslinking) was performed in this unstable period, the effectiveness, usually evaluated according to the changes of morphological and mechanical parameters, would be greatly affected. Therefore, long-term in vivo observations on variations of enzyme-mediated corneal ectasia models are needed.

Mechanical stimulation may cause changes in the stress state of the cornea, and consequently affects the corneal stroma and keratocytes. Negative pressure suction is widely used in surgical treatments, and may directly cause changes in tissue growth state as an external factor. We imagined that negative pressure suction may be helpful to accelerate the formation of corneal ectasia. Therefore, in this study, we considered two kinds of treatments on corneas, namely negative pressure suction and collagenase type II solution treatments, to observe the changes of the corneal morphological parameters and biomechanical related parameters for three months after treatment.

# Methods

## Animals

Eighteen healthy New Zealand rabbits aged 7 months were selected from the animal department of Capital Medical University. During the observation, all animals were kept in the SPF class animal room of Capital Medical University. The protocol for experimental animal was approved according to relevant laws and institutional regulations. All rabbit eyes were examined by slit lamp to exclude anterior segment

lesions. The healthy rabbits were randomly and evenly divided into three groups: the left corneas were treated with negative pressure suction (NP group), treated with collagenase type II solution (CII group), and treated with collagenase type II and negative pressure suction (CP group) in turn. All the right corneas were treated as controls. After treatments, the animals were kept for three months, during which morphological parameters and Ocular Response Analyzer (ORA) output parameters were collected. After the three months observation in vivo, the animals were euthanized by over anesthetic injection of 30mL 25% sodium pentobarbital through the ear vein for mechanical tests and histological observations.

## Morphology and biomechanical related parameters

In view of no determined evaluation criteria for the success of corneal ectasia animal model [7], we focused on increment in diopter changes, reduction in corneal thickness and biomechanical related parameters, which are regard as the signs of cornea ectasia. Keratometer (TOPCON, Japan) and Optical Coherence Tomography (OCT, TOPCON, Japan) were used to get corneal diopter along horizontal and vertical meridians (D_H and D_V). Handheld opthalmotonometer (iCare, Finland) was used to measure intraocular pressure (IOP). Ultrasound Pachymetry (TOMEY, Japan) was used to get central cornea thickness (CCT). Ocular Response Analyzer (ORA, Reichert Inc., Depew, NY) was used to evaluate the biomechanical property of cornea in vivo. The output parameters of ORA we collected are corneal resistant factor (CRF) and corneal hysteresis (CH).

## Negative pressure suction process

A home-made negative pressure device was shown in Fig. 1, which was constructed with the suction pump, 50 mL needle tubing, pressure sensor, hard connection pipe and suction catheter. The air in the suction catheter was pumped out by the suction pump, and its value of pressure was measured by the pressure sensor. According to the ideal gas state equation, $PV = nRT$, the theoretical value of pressure in the suction catheter was calculated, in which the temperature $T$ was the room temperature, R is gas constant. By comparing the theoretical and experimental values, the experimental value of pressure in the negative pressure device was calibrated.

Rabbits were anesthetized by 3% sodium pentobarbital with the dosage of 1 ml/kg, then applied to 500 mmHg pressures (260 mmHg less than one atmosphere pressure) in the left corneas with the negative pressure device for 30 minutes, once two days, three times in total. Erythromycin Eye Ointment was used twice a day as the anti-infection treatment during the first postoperative week.

## Collagenase solution treatment process

Collagenase (Gibco, U.S.) and dextran powder (Shanghai Yuanye Bio-Technology Co., China) were added to PBS solution (PH 7.4) to prepare collagenase type II solution with a concentration of 3 mg/mL, stored at -20°C against light. After anesthesia, the central area of experimental corneas was ringed with a home-made hollow tube (diameter about 6mm), and dripped with 75% medical alcohol for 30 seconds at room temperature to remove the epithelium, then rinsed with PBS solution. After that, the circular region of experimental corneas were soaked in the collagenase type II solution (200 μL) thoroughly for 30 minutes at room temperature.

Erythromycin Eye Ointment was used twice a day as the anti-infection treatment during the first postoperative week.

## Histology

At the third month after treatment, three rabbits of each group were taken out and their corneas were fixed with 4% paraformaldehyde for 24 hours, and then embedded with paraffin and stained with hematoxylin-eosin.

## Biomechanical measurements

At the third month after treatment, three rabbits of each group were taken out and their corneas were cut into a 3 mm-wide strip by a double-edged knife along the nasal-temporal direction. The uniaxial tensile test was performed on the Care-IBTC-50 Testing System (CARE Measurement & Control Corp, Tianjin, China) in normal saline bath apparatus at room temperature with 25°C. After preconditioned, the stress-strain test was carried out with the tensile rate of 0.02 mm/s, and the stretching amplitude is 115% of original length. After a 5-minute recovery, a 10-minute stress-relaxation test was performed afterwards. The mechanical parameters were calculated according to our previous study [18].

## Statistical method

Repeated measures analysis was used to analyze the biomechanical and morphological changes of the corneas across time. Paired t-test was used to analyze the collected data of experimental cornea and its controls, and to compare the collected data of pre- and post-treatment. All statistical analysis were performed using

SPSS (IBM, U.S.) with a significance cutoff of p-value at 0.05.

# Result

## In vivo tests

We collected morphological parameters (D_H and D_V, and CCT) and ORA output parameters (CH and CRF) of three groups at the first, second, third week and third month after treatment. To better describe the changes of the measurement quantities, we defined the change of them. For example, the change of CCT (ΔCCT) is as follows:

$$\Delta\mathrm{CCT} = \mathrm{CCT}_{post-treatment} - \mathrm{CCT}_{pre-treatment} \quad (6)$$

The changes of all in vivo parameters in Fig. 2 to Fig. 4 were presented by means and standard deviations.

ΔCCT of these three groups were showed in Fig. 2. Although CCT in each group fluctuated to different degrees after treatment, there was no significant overall effect for time ($p = 0.065 > 0.05$, $F = 3.153$). The peripheral corneal thicknesses of experimental eyes had the same trend with CCT. In NP group, ΔCCT of experimental eyes increased at first postoperative week (pre- and post-treatment eyes: $p = 0.006 < 0.05$, paired t-test). In CII group, CCT of experimental eyes decreased at the second week after treatment, compared with pre-treatment and the control group ($p = 0.003 < 0.05$; $p = 0.01 < 0.05$, paired t-test). Then significant difference disappeared (experimental and control eyes: $p = 0.468 > 0.05$, paired t-test) after 3 months.

The variations of diopter of the cornea along horizontal and vertical directions

(ΔD_H and ΔD_V) were shown in Fig. 3, no significant long-term change was found (p = 0.709, F = 0.47 of ΔD_H; p = 0.357, F = 1.183 of ΔD_V). In NP group, D_H was significantly decreased compared with the controls at the third week (p = 0.039 < 0.05, paired t-test). In CII group, at the first week, mean value of D_H increased 4.38D and 4.10D compared with pre-treatment and control group, respectively.

The changes of CH and CRF were shown in Fig 4. For these three groups, ΔCH and ΔCRF fluctuated around zero, the changes of mean values were lower than 1 mmHg. Repeated measures analysis demonstrated significant overall effects for time in CH (p = 0.02, F = 4.835), but not in CRF (p = 0.709, F = 0.47). In CII group (Fig. 4b), ΔCH was significantly decreased (p = 0.021 < 0.05, paired t-test) at the second week.

## Uniaxial tensile test

The uniaxial tensile test was carried out at the third month after treatment. The strain-stress curves and stress relaxation curves of corneal strips were shown in Fig 5. Following the method shown in the study [18], we divided the strain-stress curve of corneal strip into low-stress linear region, nonlinear region and high-stress linear region. The mechanical parameters gained by curves fitting were shown in Fig. 6. $E_L$ and $E_H$ were the elastic modulus of cornea at the low- and high-stress linear region, parameter $B$, the slope of tangent modulus with stress ($dE_t/d\sigma$), were obtained by exponential fitting of strain-stress curves in its nonlinear region. Stress relaxation time ($\tau$) was defined as the time over which the stress was relaxed halfway between its initial and equilibrium value [19], and relaxation limit (G(∞)) was the normalized stress

as time was infinity. The results showed that the partition fitting method can describe the strain-stress curve better ($R^2 > 0.98$), and the second order Prony model gave a good fit to the stress relaxation data ($R^2 > 0.99$). Moreover, biomechanical parameters of control corneas were basically consistent with the previous literature on healthy rabbit corneas [18].

From Fig. 6, as to the cornea treated with negative pressure suction (NP group), we noted that their elastic modulus ($E_L$ and $E_H$) increased. The stress relaxation time ($\tau$) increased compared with its controls, which means the relaxation stress became slow down, while the relaxation limit ($G(\infty)$) overlapped with its controls. In CII group, mechanical parameters are basically the same between experimental and control corneas. In CP group, only slightly differences were shown between experimental and control corneas, and such differences of most parameters were larger in CP group than those in CII group.

## Histology

In addition, HE staining was performed to observe the changes in tissue state, and to further confirm the biomechanical change of tissue. As shown in Fig. 7, the structure of each layer was intact both in experimental and control corneas, no obvious abnormality in the cell morphology and no inflammatory cell infiltration was observed, and some epithelial cells were lost due to sectioning. In NP group, there was no significant difference between the experimental cornea and its control. In CII and CP groups, compared with their control corneas, the experimental corneas tissue sections showed slightly loose and disordered collagenous fibers, widened

interlamellar clefts, and some curled fibers.

# Discussion

Corneal ectasia results in a decline in the quality of life [19], and it is a leading indication for keratoplasty [20]. Corneal ectasia animal models can be used to explore potential treatment methods, the effectiveness of which usually evaluated according to the changes of morphological and mechanical parameters. After model construction, corneas may under a series of remodeling processes, which results in an unstable state of corneal morphology and mechanical properties. Therefore, long-term in vivo observation on variations of model itself is needed. In this study, three treatments (negative pressure suction, collagenase type II solution, and treated with collagenase type II solution and negative pressure suction in turn) were performed in rabbits of three groups respectively. Then corneal morphological parameters and biomechanical related parameters were collected within 3 months after treatment to understand the long-term changes in the corneas.

In CII group, an increase of 4.38D in mean value of D_H, and decrease of CCT and CH were shown in experimental corneas 2 weeks after treatment, which is consistent with the clinical manifestations of corneal ectasia, and also consistent with Yan's result [15]. While no statistical difference in diopter was found possibly due to the individual difference or insufficient sample size. Collagenase type II can cause changes in the morphological and mechanical properties of corneas, while the characteristics of ectatic corneas were disappearing since the third week. The mean values of $E_L$ of experimental corneas were only slight smaller than those of the

controls at the third month. Histology showed some loose and disordered collagenous fibers, widened interlamellar clefts in experimental corneas, but not significant. It demonstrated that the treatment with collagenase type II can result in short-term ectatic cornea, but not last long. Enzyme can lead to fibrinolysis and a series of remodeling processes of corneal stroma. During corneal stromal remodeling, the corneal morphology and mechanical properties may be in an unstable state. If the effectiveness of treatments is studied during this period, such as the effect of collagen cross-linking on corneas, the results will be greatly affected. Therefore, further researches of the long-term corneal ectasia models are worthwhile.

In NP group, corneal thickness and diopter changed in a short period, and the elastic modulus increased and relaxation degree slowed after a three-month recovery, while histology at the third month did not show significantly change. Corneal collagen fibers are the main components and bearing structures of the cornea. HE staining in this study may not be sufficient to show the effects of negative pressure suction on corneal collagen fibrils, further researches, such as electron microscopy, may be required. In addition, we speculated that the effect of negative pressure suction on corneas may occur in two ways, one is the direct influence on the outside of the cornea, and the other is the indirect influence caused by the change of intraocular pressure [21-23]. Cornea is a biological soft tissue, the stress state of which can be changed by both of above mechanical stimulations. Then corneas may undergo a complex series of interactions of matrix and the cells, and exhibit morphological and mechanical changes. Therefore, we believed that negative pressure suction does have

a certain effect on the cornea, but may not be helpful to accelerate the formation of corneal ectasia. Further exploration of factors such as negative pressure strength and suction frequency may have positive significance for successful corneal ectasia model constructing.

CCT appeared to increase or decrease after the treatment of negative pressure suction or collagenase solution alone, respectively. As to diopter, it showed decrease or increase in NP group or CII group, respectively. Compared with treated by negative pressure suction/collagenase, the CCT and diopter in CP group seemed to show the neutralization effect of the two treatments to some extent, while CH and CRF did not show regular changes. The possible reasons we believed are as follows. ORA is used to evaluate the biomechanical behaviors of human corneas. The values of CH and CRF of rabbits are relatively small, and the sensitivity of ORA is insufficient to detect such changes. Therefore, more suitable instruments for the in vivo tests of biomechanical properties, like dynamic Scheimpflug analyzer [24], are needed in subsequent experiments. In addition, the relatively shorter repair time after wound and renewable endothelial cells of the rabbit cornea [25, 26] may be the reason of model construction failure, suitable experimental animals remain to be further explored. Furthermore, studying the biological response of the cornea to external stimulation and understanding the regulatory mechanism of corneal self-repair, may be helpful to regulate the corneal self-repair in the construction of corneal expansion model, so as to improve the success rate of model construction.

One limitation of this study was that the negative pressure suction modeling

method of NP group only considers the external factors, did not combine with the clinic. The other was the number of specimens in this study was relatively small, while we still observed significant changes at some time points in NP and CII groups. Further researches will explore the influence of the concentration of collagenase, and the strength of negative pressure suction, on the biomechanics and morphology of the cornea of rabbits or other animals.

# Conclusion

Collagenase type II results in ectatic corneas around two weeks after treatment, but the degree of ectasia decreased with time, and there was no significant difference compared with the control after 3 months. After negative pressure suction, corneal morphology changed in a short period, and elastic modulus increased and relaxation time increased after a three months recovery, indicating that the negative pressure suction do have a certain effect on cornea. The cornea ectasia induced by collagenase type II and/or negative pressure suction still needs longer observation and further study.

# Abbreviations

**NP group**: Group treated with negative pressure suction

**CII group**: Group treated with collagenase type II

**CP group**: Group treated with collagenase type II and negative pressure suction

**CCT**: Corneal central thickness

**CRF**: Corneal Resistant Factor

**CH**: Corneal Hysteresis

**D_H**: Diopter of cornea along horizontal direction

**D_V**: Diopter of cornea along vertical direction

# Declarations

## Ethics approval and consent to participate

This study was approved by the Ethics Committee at animal department of capital medical university. All the procedures adhered to the regulations of the science and technology commission of China, the regulations on the control of experimental animals and the ARVO statement on animal experiments in international ophthalmic and visual science research.

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Funding

This study was supported by National natural science foundation of China (No.31470914, 31370952). The funding agencies had no role in the research design or

conduct.

## Authors' contributions

XC acquired, analyzed data, was a major contributor in writing the manuscript. XQ performed the ORA examination. MY performed the histological examination of experimental animals. HZ and LL designed the work, interpreted the data and revised the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable.

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# Figure Legends

Fig. 1 Diagram of the negative pressure device.

Fig. 2 The change of CCT (ΔCCT) in NP group (a), CII group (b) and CP group (c). * or # represent the significant difference between experimental eyes and control eyes or pre-treatment ($P < 0.05$), respectively.

Fig. 3 The change of corneal diopter in three groups. (a-c) and (d-f) are corneal diopter along the horizontal and vertical direction (ΔD_H and ΔD_V), respectively. * represents the significant difference between experimental and control eyes ($P < 0.05$).

Fig. 4 The changes of ORA output parameters in three groups. (a-c) and (d-f) are ΔCH and ΔCRF, respectively. # represents the significant difference between post- and pre-treatment experimental eyes ($P < 0.05$).

Fig. 5 Strain-stress curves (a-c) and normalized stress relaxation curves (e-f) of corneal strips in three groups. Data were presented by mean values and standard deviations,

Fig. 6 The biomechanical parameters of cornea in three groups. (a) and (c) are the results of the elastic modulus in the low- and high-stress linear region of strain-stress curves, (b) is the results of

$d\mathrm{E}_t/d\sigma$ in the nonlinear region of strain-stress curves, (d) and (e) are the results of stress relaxation limit G($\infty$) and relaxation time $\tau$ of corneas.

Fig. 7 Hematoxylin-eosin stained corneal sections. Some slightly loose and disordered collagenous fibers, widened interlamellar clefts changes showed in CII group and CP group (the marked area).

# Figures

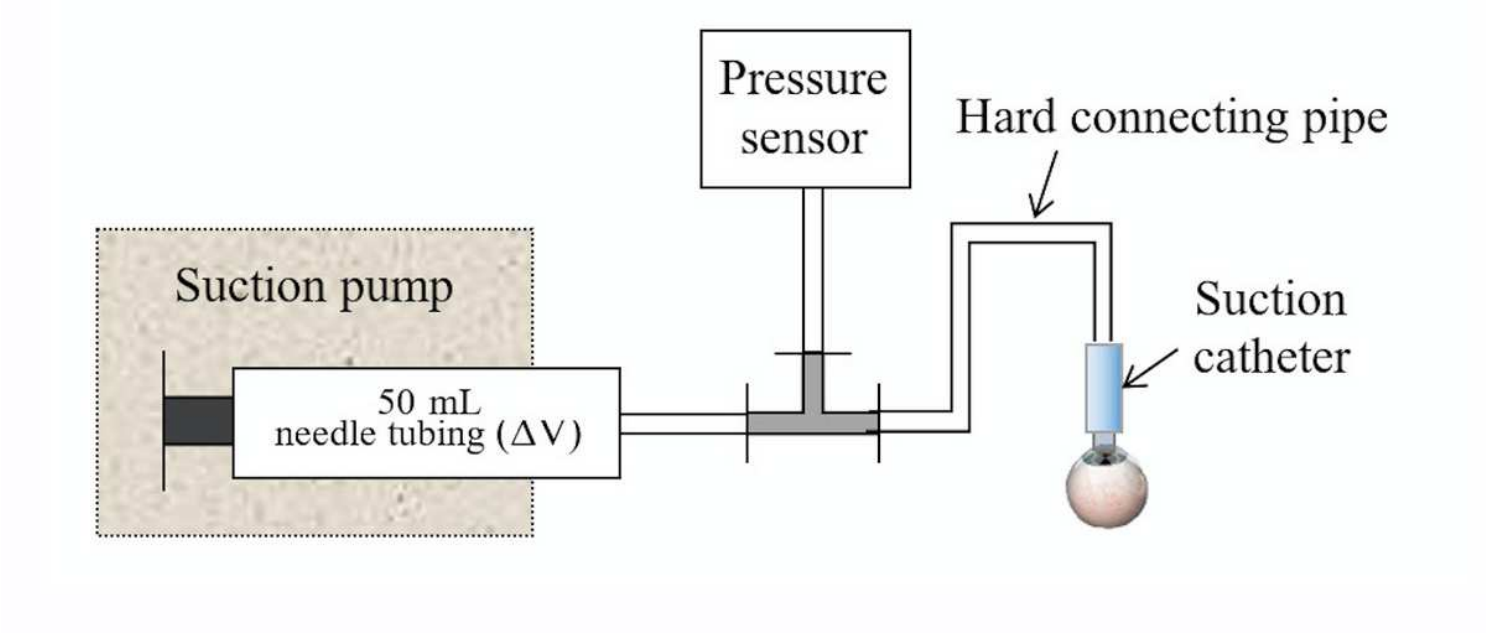



**Figure 1**

Diagram of the negative pressure device.

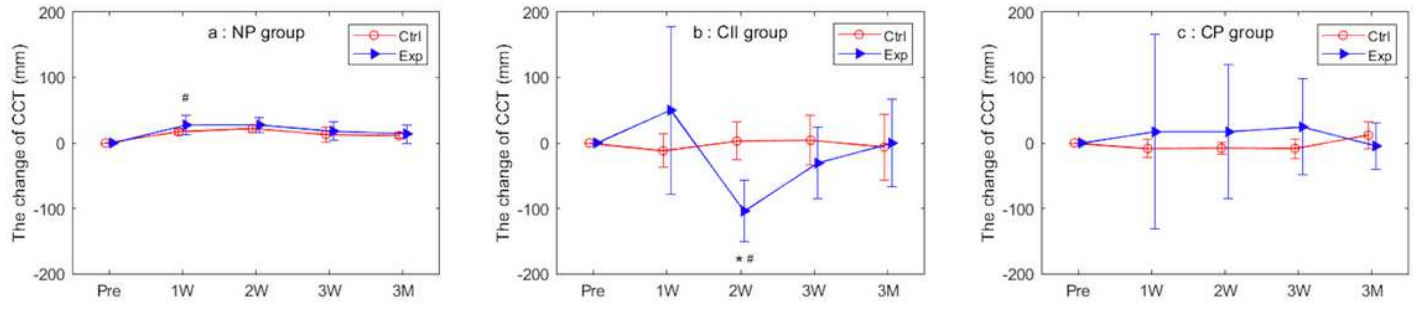
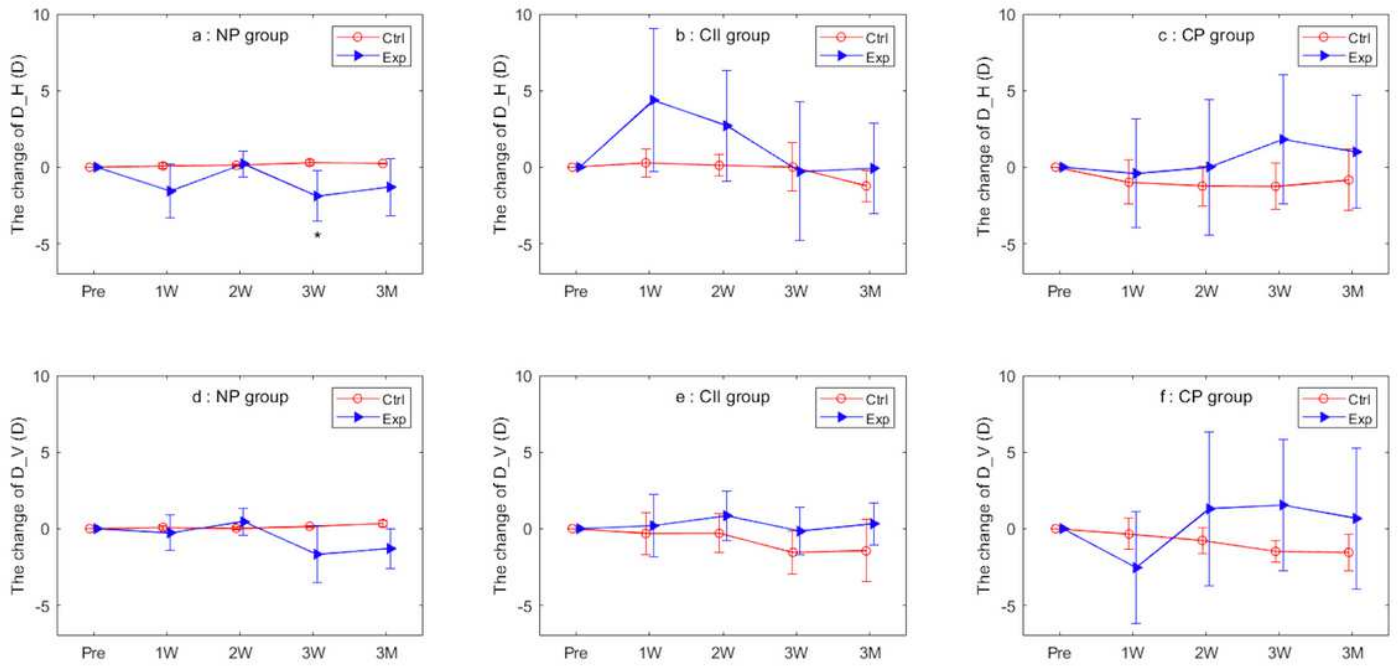


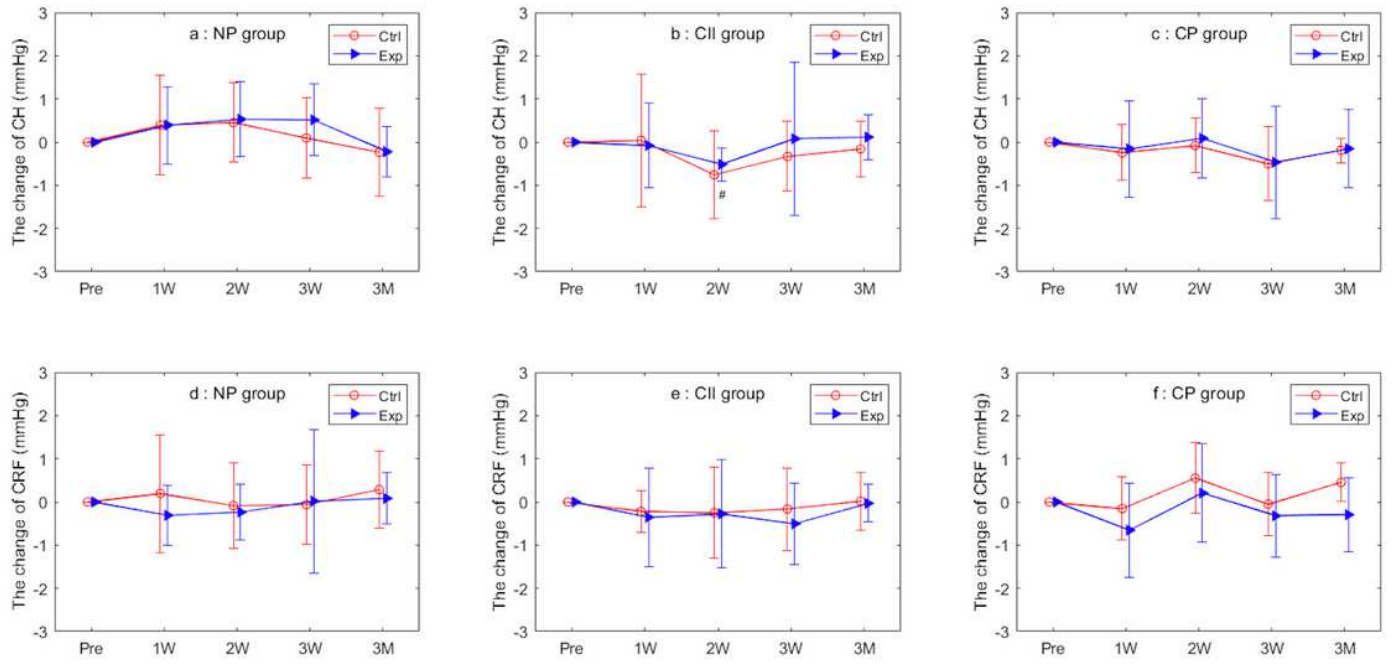

**Figure 2**

The change of CCT (ΔCCT) in NP group (a), CII group (b) and CP group (c). * or # represent the significant difference between experimental eyes and control eyes or pre-treatment ($P < 0.05$), respectively.




## Figure 3

The change of corneal diopter in three groups. (a-c) and (d-f) are corneal diopter along the horizontal and vertical direction (ΔD_H and ΔD_V), respectively. * represents the significant difference between experimental and control eyes ($P < 0.05$).




## Figure 4

The changes of ORA output parameters in three groups. (a-c) and (d-f) are ΔCH and ΔCRF, respectively. # represents the significant difference between post- and pre-treatment experimental eyes (P < 0.05).

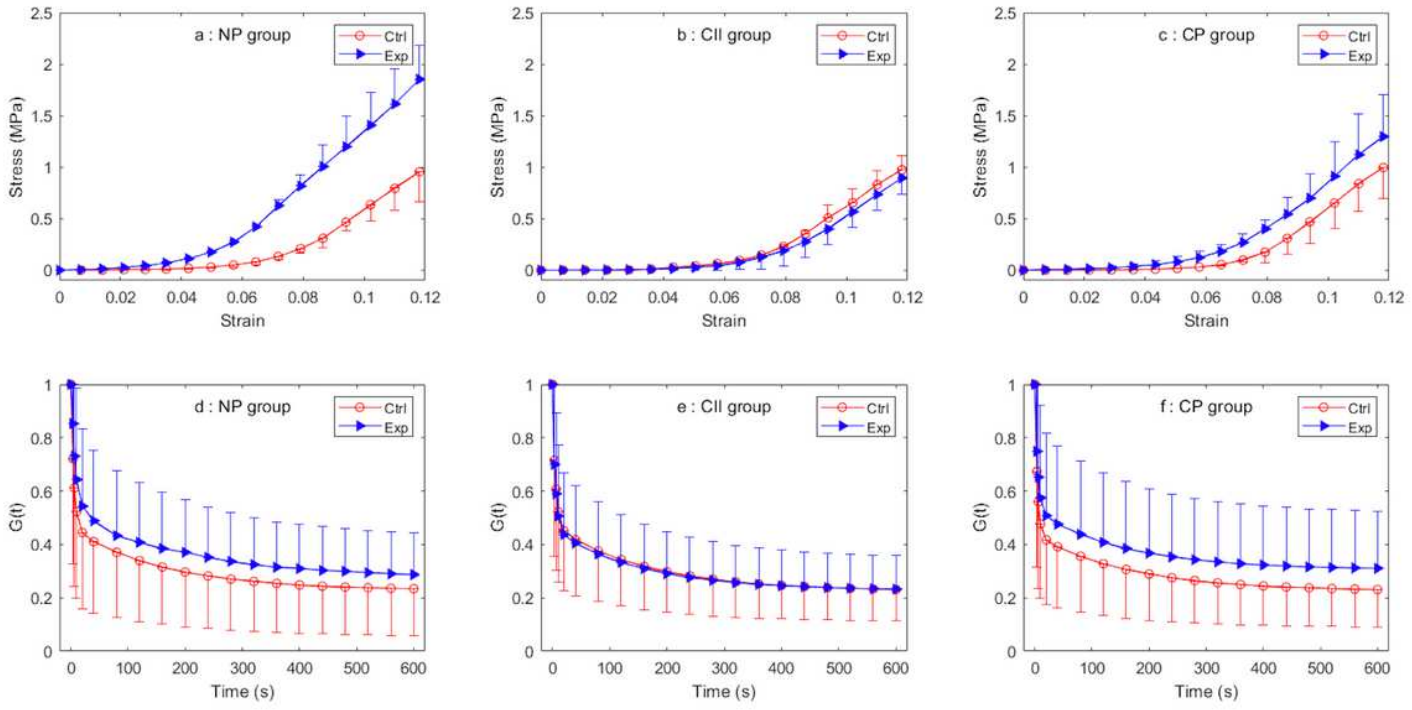



**Figure 5**

Strain-stress curves (a-c) and normalized stress relaxation curves (e-f) of corneal strips in three groups. Data were presented by mean values and standard deviations.

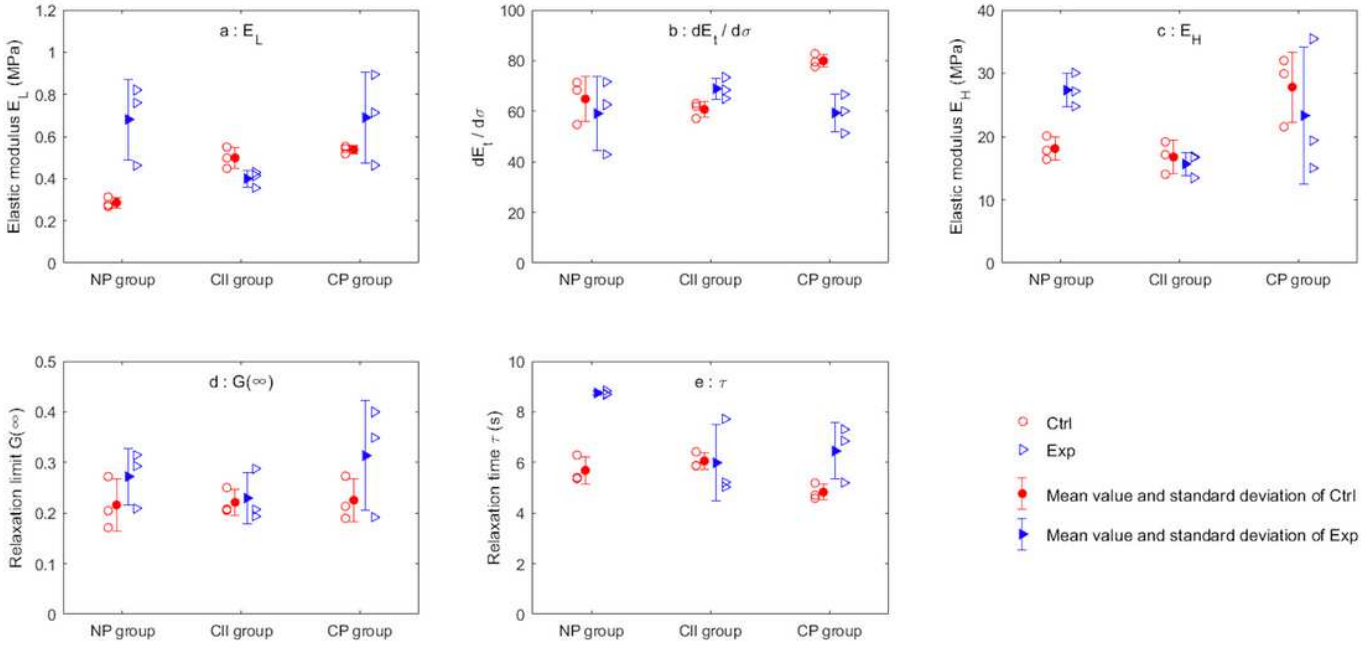



**Figure 6**

The biomechanical parameters of cornea in three groups. (a) and (c) are the results of the elastic modulus in the low- and high-stress linear region of strain-stress curves, (b) is the results of dEt/dσ in the nonlinear region of strain-stress curves, (d) and (e) are the results of stress relaxation limit G(∞) and relaxation time τ of corneas.

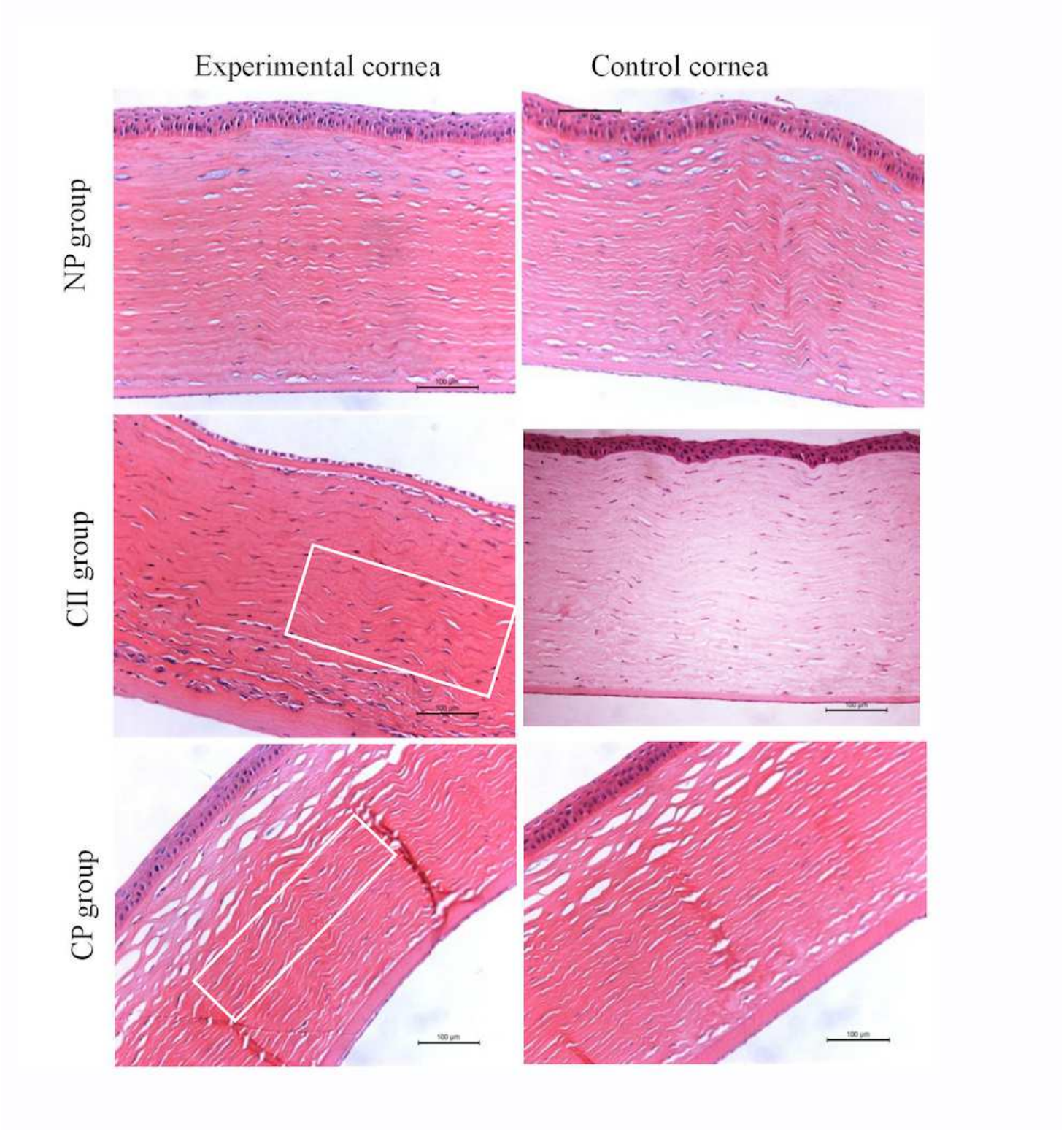



**Figure 7**

Hematoxylin-eosin stained corneal sections. Some slightly loose and disordered collagenous fibers, widened interlamellar clefts changes showed in CII group and CP group (the marked area).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsBMC.2.pdf