Efficacy of enzyme-induced collagen crosslinking on porcine cornea

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Abstract. The purpose of the present study was to investigate the effect of a new crosslinking (CXL) method, induced by enzymes, on porcine corneas. Corneal strip (10x3 mm) pairs obtained from 60 fresh porcine eyes were harvested and divided into four groups, Groups A-D. Each pair of corneal strips was incised from the central part of the same cornea; one was incubated in transglutaminase (Tgase) solution (microbial Tgase 2 produced by tissue engineering) and the other remained untreated as a control. CXL strips of Groups A-D were incubated with 2, 1, 0.5 and 0.25 U/ml Tgase solution, respectively at 37˚C for 30 min. After that, tensile strain measurements were performed for all strips. One cornea from each group was chosen randomly for hematoxylin and eosin, and Masson staining to identify histological morphology changes. The elastic modulus of treated corneas of Groups A-D were 6.56±2.93, 4.72±1.29, 5.24±2.13 and 3.48±1.60 MPa (mean ± SD), respectively at a strain of 20%, and had a 66, 43, 36 and -6% increase compared with those of their control strips. Compared with the control strips, the elastic modulus of the treated strips significantly increased in Groups A-C. The central corneal thickness of the treated corneas in Groups A-D were 1.54±0.14, 1.41±0.15, 1.47±0.11 and 1.43±0.13 μm, respectively; however, there was not a statistically significant difference compared with the control group. No reduction in corneal transparency was observed, and no obvious abnormalities were found in corneal morphology. CXL mediated by enzymes can lead to a notable enhancement in the biomechanical characteristics of the cornea while maintaining its structural integrity. Enzyme-induced CXL could be a new generation CXL method for strengthening the cornea.

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Introduction

Corneal ectasia is a group of eye disorders characterized by bilateral thinning and distortion of the central, paracentral or peripheral cornea. The primary forms include keratoconus and ectasia after refractive surgery (1). Previously, crosslinking (CXL) with ultraviolet-A and riboflavin (UVA/RF) was introduced as an effective, minimally invasive means to treat these progressive corneal diseases (2). By promoting the CXL reaction, the corneal collagenous fiber is stiffened and the deformation-resistant performance of the corneal tissue is improved (3). After the CXL procedure, astigmatism, the best corrected visual acuity, and the maximum simulated keratometry values markedly improved in patients with keratoconus (4). Clinical results showed that a flattening of ≤2 diopter within 2 years after CXL was observed and the effect of flattening was demonstrated for a period >10 years (5,6). Despite its promising results, some sequelae and complications of UVA/RF CXL, mostly related to the toxic nature of UV, have also been reported. Keratocyte loss of anterior corneal layers is inevitable (7), and endothelial cell density loss has been reported as an outcome of the thinning of the cornea after UVA CXL (8). Corneal haze has been reported in 10-90% of patients (9). Additionally, *in vivo* confocal microscopy has shown that there is an elevation in stromal reflectivity, which is indicative of edema and activation of keratocytes. This typically occurs within a timeframe of 3-6 months following treatment (10). The aforementioned issues render numerous patients unsuitable for this type of treatment.

Enzyme induced CXL, which is commonly used in the food and agriculture industry, may be a better alternative procedure. Biochemical CXL uses enzymes to induce additional peptide bonds between collagen molecules, which differs from free radical-induced CXL using photochemical CXL, such as UVA/RF CXL. Recently, the mRNA and protein levels of fibronectin and transglutaminases (Tgases) were reported to be higher in human corneal keratocytes

treated with UVA/RF CXL, so the induction of Tgases in the cornea has been proposed as a new mechanism for inducing CXL (11). In the present study, the biomechanical effects were measured and the histological changes were observed after *in vitro* Tgase-induced CXL in the porcine cornea, and the potential of this as a novel and effective method to stiffen corneal collagen was evaluated.

Materials and methods

Cornea preparations. A total of 60 fresh porcine eyes with intact and transparent corneas were purchased from the local abattoir (Beijing Shunxin Agricultural Co., Ltd., Pengcheng Food Branch; Beijing, China) <4 h postmortem. All porcine eyes were obtained from animals slaughtered for the food industry after routine slaughter. Corneoscleral rings with a diameter of 16 mm were harvested and corneal strip pairs were obtained from each cornea using a self-designed triple-blade scalpel through the corneal center, including a length of 10-mm cornea tissue and 3-mm sclera on the bilateral ends. A pair of corneal strips from the same cornea was distributed randomly into two groups: One was incubated in 1,500 U/g Tgase solution (CAS no. 80146-85-6; Hefei Bomei Biotechnology Co., Ltd.), and the other one was incubated in PBS and used as a control.

CXL procedure. A total of 60 corneal pairs were divided into four groups, Groups A-D. CXL strips in Groups A-D were incubated in Tgase solution, with physiological saline solution was used as the solvent. After full dissolution, the CXL agent solution was formed. The tissue material was immersed at 37˚C for 30 min in 2 U/ml Tgase solution (Group A; n=15), 1 U/ml Tgase solution (Group B; n=15), 0.5 U/ml Tgase solution (Group C; n=15) or 0.25 U/ml Tgase solution (Group D; n=15). A total of 60 untreated corneal strips (15 per group), which were paired with CXL-treated strips, were placed in PBS at 37˚C for 30 min. The temperature of a normal human body varies between 36.1 and 37.2˚C (12). Incubation was carried out at 37˚C, as this temperature is close to the temperature of the human body. Additionally, as the enzyme is found naturally and is functional in the human body, a temperature of 37˚C was considered appropriate for optimal performance. The corneal materials were then separately stored in PBS at room temperature (25˚C). All corneal strips were used for subsequent biomechanical measurements 0-3 h after CXL.

Corneal morphology and thickness. After CXL, the transparency and morphology of a hemisphere of corneal material were observed and recorded. The thickness and width of every corneal strip were measured using a micron-thickness gauge (Yiwu Exploit Hardware Co., Ltd.) and a Vernier caliper.

Biomechanical assessment. The corneal strips were clamped vertically in the tongs of an electronic universal testing machine (Shimadzu Corporation) with the sclera and corneoscleral tissue both held in the tongs at both ends. The tensile strain test was run at 2 mm/min the initial strain loading to 0.05 N, with the length of the corneal trips between both tongs recorded as the initial length (Fig. 1). The elastic modulus was calculated using the software included with the testing machine (Trapezium; version 1.5.1; Shimadzu Corporation).

Histological staining. Three samples were randomly selected from each group for morphological observations. These samples were fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd.) at room temperature for 24 h. The main body of the three corneal materials was used to create corneal test strips, while the remaining parts of the cornea were subjected to hematoxylin and eosin (H&E) and Masson staining. For H&E staining, the prepared sections were immersed in a series of graded ethanol baths followed by xylene to dehydrate the tissues. The thickness of the sections were 4-μm. After dehydration, the tissues were immersed in paraffin. They were then stained with H&E (hematoxylin 2 min; eosin 1 min) before examination. For Masson trichrome staining, the prepared sections were immersed in hematoxylin (5 min), scarlet-acid (5 min) and aniline blue (1 min) solutions successively, with 70% ethanol used for dehydration between the stains. The thickness of the sections were 4-μm. All staining steps were performed at room temperature (25˚C). All images were captured using a light microscope at x200 magnification with a high-resolution charge-coupled device camera. The images were analyzed using NIS-Element imaging software (version 3.2; Nikon Corporation). Changes in histological morphology between the CXL and control group were assessed.

Statistical analysis. The exponential fitting of the stress-strain curves was produced using OriginPro (version 2018; OriginLab Corporation). Data for treated and control cornea materials were compared using an independent sample t-test in SPSS (version 24; IBM Corp.). The distribution of data between the different groups was investigated using the Kruskal-Wallis test. Data are presented as mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tangent gradients in the stress-strain curves become steeper after CXL. The stress-strain curves showed a marked increase in the biomechanical properties (elasticity modulus) of treated corneas in a concentration-dependent pattern. Compared with the control groups, the tangent gradients in the stress-strain curve of the treated group were steeper at higher concentrations of Tgase (Group A, B and C). These differences were statistically significant in Group A, B and C. Although the stress-strain curve of the control group in the lower Tgase concentration group (Group D) seemed to be steeper than that of the experimental group, there was no significant statistical difference between the two groups (Fig. 2A and Table I).

Elastic modulus of the cornea increases after CXL. As a nonlinear material, the elastic modulus of the cornea strips was altered under different strains and the changes in elastic modulus were different in the four groups. Differences were particularly notable in the 10-20% strain measurements (Fig. 2B and C). The elastic modulus of the CXL-treated corneas and their corresponding controls were calculated and

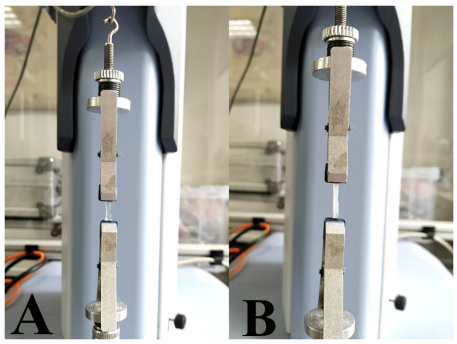

Figure 1. Schematic diagram for measuring the elastic module of the corneal strips. Corneal strips clamped on the testing machine (A) before and (B) after the tensile strain test.

listed at 20% strain (Table I). The average increase in elastic modulus in the different concentration CXL subgroups (2, 1 and 0.5 U/ml) was 166, 143 and 136%, respectively, compared with those in the control group. These differences were found to be statistically significant (Fig. 2D). There was no significant difference in the distribution of elastic modulus among the control subgroups at different concentrations (P=0.591).

Thickness of the cornea remains unchanged. The central corneal thickness of the CXL strips and controls were assessed (Fig. 2E). The corneal thickness of the experimental group (Tgase-treated cornea strips) showed a trend of thinning compared with the control group at the following Tgase concentrations: 2, 1 and 0.5 U/ml, but the difference was not statistically significant (Table II).

Transparency of the cornea remains unchanged. Transparency was similar in the CXL cornea strips (Fig. 3A, left) and control strips (Fig. 3A, right).

Stiffness of the cornea becomes stronger after CXL. The stiffness and deformation resistance of corneal strips in the CXL groups (Fig. 3B) were better than those in the control groups (Fig. 3C).

Histological structure of the cornea is not damaged. H&E staining showed that the overall structure of the cornea was not damaged after Tgase-induced CXL treatment. Masson trichrome staining showed that the arrangement of fibrils in the CXL groups was as ordered as that in the control group. The interfibrillar space was similar in both the CXL and control groups (Fig. 4).

Discussion

The present study used elasticity modulus to investigate the changes in the corneal biomechanical properties after enzyme-induced CXL. The results of the present study indicated that the corneal biomechanical property (elasticity modulus) was significantly enhanced by enzyme-induced CXL and that the increase was dose-dependent. CXL solutions concentrations of 2, 1 and 0.5 U/ml resulted in increases in the elastic modulus of 166, 143 and 136%, respectively. It was shown that the increase in the Young's modulus produced by Tgase was between 1.36 and 1.66x, depending on the concentration. In the current study, a decrease was observed in the elastic modulus of the materials when using the lowest concentration of Tgase (0.25 U/ml) for CXL. However, these observed differences could likely be attributed to measurement errors. Initially, the elastic modulus was expected to remain relatively stable. Measurements were performed using an electronic universal testing machine and the research outcomes were sensitive to environmental conditions. Factors such as room humidity during material testing, the moisture content of the tissue and the duration of tissue detachment all serve a marked role in influencing the measurement results (13). Therefore, if the changes are statistically insignificant, it can be concluded that the material properties remain unaffected. Due to the lack of statistical differences observed in the low concentration group (0.25 U/ml), we hypothesize that enzyme-induced CXL therapy may require the enzyme to reach a certain concentration in order to achieve therapeutic effects.

Cornea, as a nonlinear material, had no directly proportional relationship of stress to strain. The relationship of stress vs. strain is often used to reflect the biomechanical characteristics of the material. At a moderate-to-high enzyme concentration, the tangent gradients in the stress-strain curve of the treated group were steeper compared with the control groups. The modulus-strain plots also showed that the biomechanical nature (elasticity modulus) of the treated corneal strips in the 2, 1 and 0.5 U/ml concentration groups were significantly increased compared with the controls at a high strain (10-20%) after CXL. All the curves and elastic moduli showed no significant difference at a low Tgase concentration (0.25 U/ml). This finding indicated that the effective concentration should not be <0.5 U/ml in future clinical practice. However, increases in values might be lower *in vivo* because the enzyme solution may not be able to penetrate the cornea and only the anterior half of the cornea could be crosslinked. In the present study, the whole cornea including both epithelial and endothelial surfaces was crosslinked, which should increase the CXL effectiveness. Compared with the 2 U/ml group, the 1 U/ml group reduced the dosage of the drug by half, while the effective value only decreased slightly. Therefore, 1 U/ml may be a more appropriate dose *in vivo*.

Compared with that in the control group, the thickness of the cornea in the treatment group demonstrated a trend of decrease after CXL at a moderate-to-high concentration; however, there was no statistically significant difference. It has been reported that thickness was reduced after UVA/RF CXL (14-16). The decrease may occur at the end of the CXL operation (11) and continue for ≥1 year (15,16). Anatomical and microstructural changes in corneal collagen fibrils, such as the compression of collagen fibrils, may change corneal hydration and edema (15). Further research is required to confirm this. In the present study, the transparency of corneas treated with Tgase-induced CXL was found to be comparable to that in the control group when observed with the naked eye. Additionally, there were no observed changes in the structure of the corneal collagen fibers when examined under a light microscope. Histological observations of the fibrils showed no noticeable alterations after Tgase-induced CXL treatment, and the orientation and separation of collagen lamellae were consistent across the different concentration groups. These findings suggested that

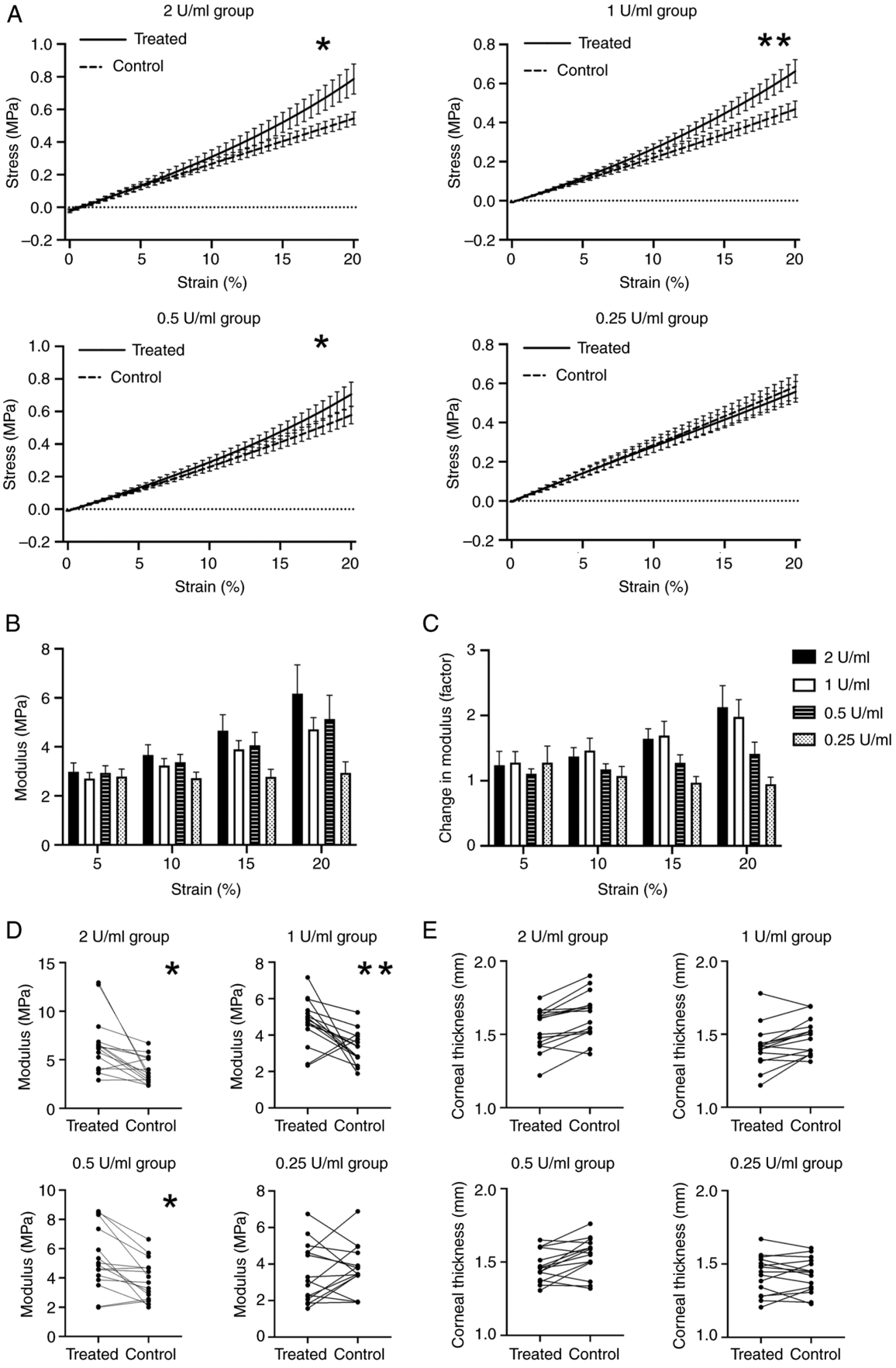

Figure 2. Changes in the stress-strain curves, elastic modulus and thickness of the corneal strips after Tgase-induced CXL. (A) Stress-strain curves with standard error bars for Tgase-induced CXL treatment of cornea strips and control corneas at different enzyme concentrations (n=15 per group; mean ± SE). Elastic modulus of porcine sclera strips under different strains after Tgase-induced CXL treatment of cornea strips at different enzyme concentrations; (B) raw modulus and (C) increase factor of modulus. Distribution of the (D) elastic modulus and (E) corneal thickness at different enzyme concentrations. A, D and E, were analyzed using an independent sample t-test. *P<0.05. **P<0.01. Tgase, transglutaminase; CXL, crosslinking.

Table I. Effect of different enzyme concentrations on elastic modulus in porcine corneas.

	Elastic modulus, MPa			
Groups	Treated groups	Control groups	t-value	P-value
A	6.56±2.93	3.94±1.35	2.557	0.016
B	4.72±1.29	3.30±0.91	3.449	0.002
C	5.24±2.13	3.85±1.40	1.852	0.049
D	3.48±1.60	3.72±1.31	-0.447	0.658

Group A (Tgase, 2 U/ml; n=15), Group B (Tgase, 1 U/ml; n=15), Group C (Tgase, 0.5 U/ml; n=15), Group D (Tgase, 0.25 U/ml; n=15) and Control (PBS; n=15 per treatment group). Data are presented as mean ± SD. Tgase, transglutaminase.

Table II. Central corneal thickness after enzyme-induced crosslinking in porcine cornea.

	Mean corneal thickness, μm			
Groups	Treated groups	Control groups	t-value	P-value
A	1.54±0.14	1.63±0.16	-1.883	0.069
B	1.41±0.15	1.48±0.12	-1.412	0.169
C	1.47±0.11	1.54±0.13	-1.547	0.136
D	1.43±0.13	1.42±0.12	0.064	0.950

Group A (Tgase, 2 U/ml; n=15), Group B (Tgase, 1 U/ml; n=15), Group C (Tgase, 0.5 U/ml; n=15), Group D (Tgase, 0.25 U/ml; n=15) and Control (PBS; n=15 per treatment group). Data are presented as mean ± SD. Tgase, transglutaminase.

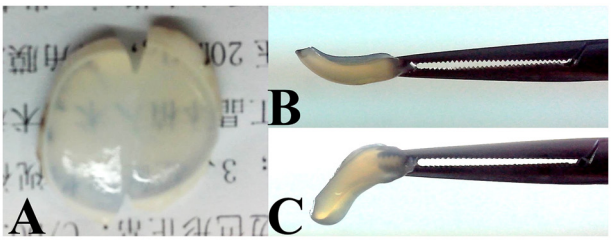

Figure 3. Morphology of the cornea. (A) Morphology of cornea (left) after enzyme-induced crosslinking and (right) control cornea. Appearance of (B) treated and (C) control corneal strips.

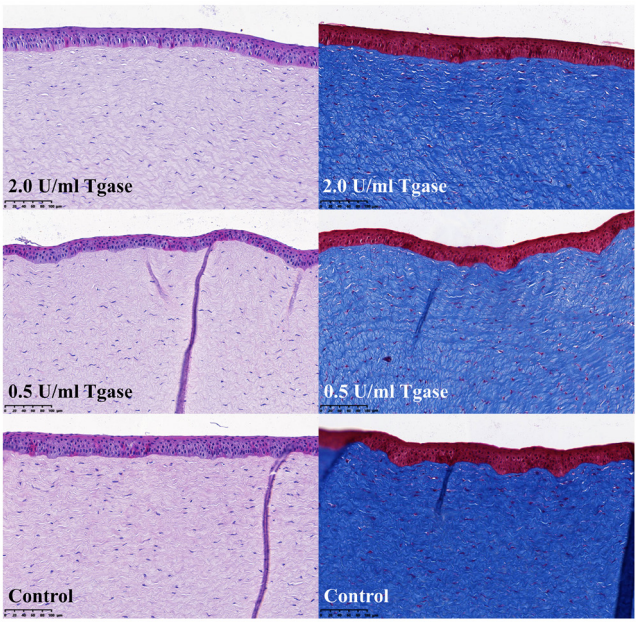

Figure 4. Histological staining of the corneal tissues. Corneal tissues imaged using an optical microscope using (left) hematoxylin and eosin staining and (right) Masson's trichrome staining from corneal crosslinking groups at different enzyme concentrations and control groups. Tgase, transglutaminase.

Tgase-induced CXL may be safe for future *in vivo* applications. However, further investigation is needed to determine if there are any changes at a smaller scale, such as changes at the molecular level. All scleral tissues were fixed in formaldehyde before H&E and Masson's staining; however, formaldehyde is also a chemical CXL agent and it may present false-negative results in the morphological analysis (17). Additional studies are needed to evaluate the changes in the diameter of collagen fibers and the interfibrillar spacing of stromal collagen fibrils using electron microscopy.

Tgases are considered a widespread enzyme family and are present in numerous different cell types and tissues with diverse functions, such as programmed cell death, cell adhesion, and interaction between the cell and the extracellular matrix through the CXL of proteins (18). The mechanism underlying CXL induction by Tgases is the formation of isopeptide bonds between the γ-carboxamides of glutamine residues (donor) and the first-order e-amine groups of different compounds (19). In the past, commercial Tgases could be obtained only from animal tissues, such as guinea pig livers, with the low yields and high price preventing Tgases from being more widely applied. Tgases may now be obtained from microorganisms at increased yields and this allowed a number of novel potential applications to be developed using Tgases (20). In the food industry, it can be used for protein modification, while in the biotechnology and pharmaceuticals, it can be used to mediate bioconjugation (21). Although Tgases are enzymes that are widely distributed throughout the human body, they are scarce in the cornea because of their lack of blood and lymphatic vessels. The cornea consists almost exclusively of type I collagen and is rich in glutamic acid and lysine. In theory, corneal collagen fibers could be crosslinked by Tgases, potentially resulting in more resistant mechanical properties (22). Previously, the mRNA and protein expression levels of fibronectin and Tgases were reported to be increased in human corneal keratocytes following UVA/RF CXL treatment, and the induction of Tgase in the cornea was proposed as a novel mechanism for inducing CXL (11).

Tgases are widely used in various fields, including the food and manufacturing industries. The induction of Tgases has been suggested to have a low toxic potential and be safe for the human body (23). At the same time, enzyme-induced CXL works as a direct CXL method and does not require UV irradiation. The cytotoxicity of UVA may produce a highly

active oxygen species that causes oxidative stress and induces necrosis or apoptosis in keratocytes (7). Currently, UVA/RF CXL is the only clinically approved CXL technique, but it is unsuitable for corneas <400-μm thick due to cytotoxicity (24). The disadvantages of cytotoxicity in UVA/RF CXL may be avoided by Tgase-induced CXL. Previous studies have shown that Tgase-induced CXL does not cause damage to the endothelium and keratocytes in the cornea (25). Therefore, Tgase-induced CXL is expected to become a new generation of CXL used in corneal ectasia. Nevertheless, direct administration of Tgases as eye drops into the conjunctival sac may not be feasible. To the best of our knowledge, there are currently no studies that have directly administered Tgases as eye drops into the conjunctival sac. The dilution by tears and its potential toxic side effects on nearby tissues may need to be considered. Possible routes of administration required further investigation in future studies.

To the best of our knowledge, no studies on the effect of Tgase-induced CXL on the cornea have been previously reported. The findings of the present study indicate that Tgases markedly increase the stiffness of the cornea. Enzymatic/biochemical CXL has a large number of unknown and technical points to be investigated. However, it is possible that in the future, Tgases could be developed as pharmaceutical agents to modify the biomechanical properties of the cornea, which could potentially slow down or treat keratectasia and provide an optimal treatment approach for conditions such as keratoconus. Furthermore, the ocular surface typically has a temperature of ~34˚C. In future clinical use, the temperature range is anticipated to be 34-37˚C. Further research is required to investigate the safety, enzyme concentration, enzymatic reaction time, long-term efficacy and other aspects of enzyme-induced corneal CXL *in vivo* and *in vitro*.

In conclusion, enzyme-induced CXL should be investigated further and is expected to be a new generation CXL method which could be used in corneal ectasia.

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Availability of data and materials

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

Authors' contributions

YW conceived and designed the study. SZ, WZ, SX, YZ, DC and XL performed the experiments and collected the data. DC and XL finished the uniaxial tensile test and calculated the Young's modulus of materials. YW contributed to the data analysis. SZ and WZ contributed equally to the manuscript writing. YW and SZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved (approval no. J2022103) by the Laboratory Animal Ethics Committee of the First Hospital of Peking University (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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