Influence of microstructure on stiffening effects of corneal collagen crosslinking

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Running Title: Crosslinking of posterior/anterior corneal flaps

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Abstract

Background/Objectives: The corneal collagen crosslinking (CXL) has become a new treatment procedure for stopping the progression of keratoconus. This treatment procedure has widely been studied in order to optimize its commonly used protocols and improve patient comfort. Nevertheless, many of these studies were not successful in clinics because the exact underlying mechanisms of this procedure are not still fully understood. The present study investigates the stiffening effects of CXL on tensile properties of anterior and posterior corneal flaps in order to provide new data on the working principle behind this treatment option.

Methods: A DSAEK system was used to prepare anterior and posterior flaps from porcine corneas. The flaps were subjected to UVA/riboflavin collagen crosslinking treatment and their mechanical behavior was assessed by conducting uniaxial tensile experiments. Furthermore, full thickness corneas were crosslinked from the posterior and anterior side and their tensile behavior was measured.

Results: It was found that CXL procedure significantly improved the biomechanical properties of the anterior flaps ($p<0.05$). Nevertheless, it did not have any significant effect on the tensile properties of posterior flaps. Furthermore, it was observed that crosslinking full thickness porcine corneal stroma from the posterior part had no significant stiffening effect.

Conclusions: The stiffening effect of the collagen crosslinking therapy significantly depends on the composition and microstructure of corneal extracellular matrix.

Introduction

Keratoconus is a progressive eye disease in which the cornea thins and starts to become conical in shape. Although the etiology of this eye disease is not fully known, it significantly reduces the mechanical strength of the tissue [1]. Corneal collagen cross-linking (CXL) procedure is a relatively new treatment which is currently used to halt the progression of this eye disease [2, 3]. This therapeutic intervention uses the photosensitizer riboflavin solution and ultraviolet A light (UVA) to enhance the mechanical properties of the cornea by inducing cross-links in corneal extracellular matrix. There has been great progress in characterizing the effect of this treatment option on corneal hydrodynamic behavior, collagen fibril diameter, keratocytes, and endothelial cells among others [4-8]. Nevertheless, its exact molecular mechanisms are not fully understood. A complete understanding of the working principle of riboflavin/UVA collagen crosslinking therapy is crucial for being able to propose new modified protocols for this treatment option [9-14].

The mechanical properties of the cornea are mainly dependent on its extracellular matrix (stroma), which makes up about 90% of its thickness and includes the majority of collagen and proteoglycan content of the tissue [15-17]. Inside the stroma, collagen fibrils are organized into 1-2 um thick sheet-like lamellae, which show a depth dependent organization, i.e. the anterior lamellae are interwoven while the posterior ones are arranged parallel to the surface. In addition to the inhomogeneous microstructure of the corneal stroma, it has been shown that the riboflavin solution uptake is limited to the anterior stroma [18]. Thus, it has been hypothesized that the collagen crosslinking therapy should have an inhomogeneous stiffening effect over the corneal thickness. This hypothesis has been tested before and been proven true by characterizing the stiffening effect of collagen crosslinking in different depths of the stroma. To the best of our

knowledge, all of these previous studies have been done by crosslinking full thickness corneas [8, 19-22].

In the present study, anterior flaps and posterior flaps were excised from porcine corneas and were crosslinked separately in order to determine possible stiffening effects of cross-linking with riboflavin and UVA on posterior and anterior flaps. Although crosslinking of posterior flaps may not be common in vivo, it will provide new data on the collagen crosslinking procedure and can be considered as a step forward to better understand the mechanisms involved in collagen crosslinking and could assist researchers who are working in modifying CXL clinical protocols.

Materials and Methods

This study used porcine eyes from a local slaughterhouse. A DSAEK system was used to excise anterior corneal flaps from corneoscleral rings obtained from the eyes, Figure 1. The thickness of all corneal samples was brought to 800 um before cutting the flaps. The thickness of the anterior flaps and the remaining posterior portion was measured by a digital pachymeter (DGH Technology Inc., Pennsylvania) immediately after dissection. 5 mm wide nasal-temporal strips were prepared using a double-bladed cutting device from anterior and posterior flaps.

Both anterior and posterior strips were soaked in photosensitizer solution composed of 10 mg riboflavin-5-phosphate in 10 mL 10% dextran T500 until their thickness reached equilibrium. Higher concentration of dextran (20%) was also tried and similar results were found. During this period, the thickness of strips was measured occasionally using the pachymeter. The strips were

placed on plastic semi spheres and were subjected to a UVA irradiance of 3 mW/cm^2 for 30 minutes. The UVA light (370 nm) source was at a distance of about 2 cm from the samples and drops of photosensitizer solution were continuously applied to the cornea during the treatment period.

The crosslinked strips were immediately mounted in a DMA machine (TA instruments, Maryland) after measuring their thickness. First, 20 mN tare load was applied to remove any slack. The displacement rate was 2 mm/min and the samples were stretched to 10% strain. The experiments took less than a minute to complete; thus no bathing solution was used (Note the thickness was measured after the experiments and no significant dehydration was observed, i.e. no hydration effect on mechanical measurements is expected [23, 24]). The stress-strain was plotted in order the compare the behavior of different groups. One way analysis of variance with a significance level of 0.05 was used to compare statistically the experimental data.

Results

Figure 2 shows the stress strain response of anterior and posterior flaps. Comparing the behavior of anterior and posterior flaps from the control group shows that anterior corneal flaps had a stiffer tensile response compared to that of the posterior ones ($p<0.05$). Furthermore, it was found that the crosslinking treatment increased the tensile properties of the anterior flaps ($p<0.05$). However, it had an insignificant effect on the biomechanical properties of the posterior flaps. Figure 3 reports the maximum tensile stress and tangent modulus of the flaps from the

crosslinking and control groups. For the anterior groups, both the stress and the tangent modulus increased significantly after collagen crosslinking therapy ($P<0.05$).

Discussion

Collagen crosslinking with riboflavin and ultraviolet A light is a relatively new treatment option to arrest the progression of keratoconus. In this work, we investigated the effect of the collagen crosslinking therapy on the biomechanics of flaps obtained from the anterior and posterior regions in order to provide more data on possible molecular mechanisms responsible for corneal collagen crosslinking.

McCall et al. found that the presence of carbonyl groups and reactive oxygen species are necessary in cross-linking treatment [25]. The formation of crosslinking in the corneal stroma requires reactive oxygen species, which are created when UVA photosensitizes riboflavin [26]. Zhang et al. showed that collagen crosslinking procedure creates crosslinks between collagen molecules themselves as well as between core proteins of the proteoglycans [26]. Nevertheless, strong crosslinks between collagen and proteoglycan core proteins have not been observed. Furthermore, Hayes et al.'s study suggested that cross-links should mainly occur at the surface of collagen fibrils and in the proteoglycan network surrounding them [4].

Figure 2 showed that the collagen crosslinking procedure significantly improved the mechanical properties of the anterior flaps but it had little effect on the tensile properties of the posterior flaps. First it is noted that the results in this plot showed that anterior flaps had much stiffer

136 tensile properties compared to the posterior flaps, which is in agreement with previous studies.
137 Randleman et al. found that the anterior stroma had significantly higher cohesive tensile strength
138 than the posterior stroma [27]. Scarcelli et al. used Brillouin Optical Microscopy in order to show
139 that anterior portion of the stroma has the highest elastic modulus in the cornea [28]. Indentation
140 techniques were also used to show that the Young's modulus of anterior stroma was significantly
141 larger than the Young's modulus of posterior stroma [29, 30]. Furthermore, Kohlhaas et al. reached
142 the same conclusion by running uniaxial tensile experiments on posterior and anterior flaps [19]. It
143 is noted that these previous studies captured the depth dependent corneal mechanical property;
144 nevertheless, the actual qualitative values vary from one study to another because of different
145 species, experimental protocols, and techniques that have been used.
146
147 The corneal stroma is composed of collagen fibrils embedded in a proteoglycan matrix. The
148 proteoglycans (PGs) are attached to the collagen fibrils through their core proteins while the
149 interaction between their negatively charged glycosaminoglycan (GAG) side chains holds the
150 collagen fibrils at the quasi-uniform spacing. The collagen fibrils are organized into 1-2 um thick
151 sheet-like lamellae, which are stacked parallel to the surface of the cornea. The arrangement of
152 the lamellae changes through the thickness and anterior lamellae interweave markedly more than
153 the posterior ones [15, 17]. This inhomogeneous architecture of the corneal stroma affects its
154 biomechanics such that anterior layers show much higher elastic modulus than the posterior
155 portion of the stroma [19, 27-30].
156
157 Previous studies have exclusively focused on characterizing the stiffening effect of collagen
158 crosslinking when full thickness corneas were used, which replicates what is done in clinics. The

159 commonly used crosslinking protocol has been designed such that it affects primarily the anterior
160 300 um of the cornea in order to avoid UV light damage to endothelial cells. Thus, it is natural to
161 expect the stiffening effect of collagen crosslinking procedure to be depth dependent, too. There
162 have been various studies in the literature confirming this conclusion. Brillouin microscopy of
163 collagen crosslinked samples showed that anterior portion of the stroma accommodated the
164 majority of the mechanical stiffening [20]. Mechanical tests such as uniaxial tension and indention
165 testing on samples excised from anterior and posterior crosslinked stroma showed crosslinking
166 caused a significant increase in anterior stroma stiffness but an insignificant change in posterior
167 stroma stiffness [19, 29]. Indirect methods have also been done to reach the same conclusion [8, 22].
168 For instance, it was found that keratocyte apoptosis was primarily located in the anterior stroma
169 when the usual surface irradiance of 3 mW/cm^2 was used [8]. These previous studies discussed the
170 depth dependent collagen crosslinking primarily in terms of the absorption behavior of the
171 riboflavin-treated cornea for UVA. Kohlhaas et al.'s study showed that about seventy percent to
172 of UVA irradiation was absorbed within the anterior part of the cornea [19]. Furthermore,
173 Sondergaard et al. determined the riboflavin distribution in the corneal distribution and
174 concluded that riboflavin uptake is limited to the anterior layers independent of the concentration
175 and application time of the riboflavin solution [18]. The astonishing finding of the present study
176 was that collagen crosslinking therapy did not improve the tensile property of posterior flaps.
177
178 The imaging techniques have clearly shown that there are distinctive differences between the
179 collagen lamella organization in the anterior and posterior stroma, i.e. significantly more
180 intertwining of fibers exists in the anterior layers. In addition, electron micrographs of normal
181 corneal samples showed that the density of collagen fibrils in the anterior stroma was

significantly larger compared to the posterior stroma. Nevertheless, the density of proteoglycans was larger in the posterior stroma. The increase in concentration of proteoglycans in the posterior layers has been used to explain the larger center-to-center collagen inter fibrillar spacings in the posterior stroma. It is also proposed that keratoconus disease involves overproduction of proteoglycans, which enhances disorganization and slippage of the collagen lamellae. Considering that previous studies suggested that crosslinks are either at the surface of the fibrils or within the proteoglycan matrix surrounding them, it is not clear why collagen crosslinking did not stiffen the posterior flaps. Here, in order to collect additional data on this confusing finding, we crosslinked addtional five whole porcine corneal stromas from the endothelium side (Group A) and five whole thickness stromas from the epithelium side (group B). We mechanically measured the tensile behavior of these samples and compared them with the tensile properties of five control samples (Group C). Control samples were subjected to the same treatment as specimens in groups A and B except that the UV light was turned off during the treatment procedure. The crosslinking therapy and the uniaxial tests were conducted as they were described in the Materials and Methods section with the only difference that we used full-thickness corneas here. Figure 4 compares the mechanical response of these three groups. It is seen that the collagen crosslinking procedure significantly improved the mechanical properties of full thickness porcine corneas only when these samples were treated from the anterior side. We also did another study in which we crosslinked 5 anterior flaps from the rear side following the exact procedure that described in previous sections (results are not shown). It was found that the collagen crosslinking had a similar stiffening effect on these samples as it did on those which were crosslinked from their top surface (epithelium side). The present study clearly showed that the mechanical testing method, which is normally used to prove the success of collagen

crosslinking treatment and its various alternation, did not show any stiffening of these layers when they were subjected to this treatment procedure. Future studies are required to use alternative methods and investigate whether crosslinks occur in the posterior layers in order to be able to fully explain the underlying mechanisms. Nevertheless, we can still provide an explanation for the observed behavior using the results of previous studies stating that crosslinks mainly occur at the collagen fibril surface and in the protein network surrounding the collagen fibrils [4].

The corneal stroma is composed of collagen fibrils and proteoglycan matrix. Recent studies suggested that proteoglycans behave as interfibrillar spacers and connect to the neighboring collagen fibrils through their core proteins [31, 32]. They interact with each other by their highly sulphated glycosaminoglycan side chains and form a soft hydrophilic coating around the fibrils. Despite being negatively charged, if they are held close to each other, glycosaminoglycans could form supramolecular organizations and act as structural bridges between neighboring fibrils [31, 32]. The corneal extracellular matrix can be modelled as a composite material composed of collagen fibril reinforcements and a hydrated proteoglycan matrix [33]. Collagen crosslinking therapy increases the mechanical properties of the proteoglycan coating surrounding the collagen fibrils. However, the relative distance between the GAG side chains is not short enough for them to be entangled sufficiently. Thus, collagen crosslinking causes an insignificant increase in the biomechanical properties of the posterior lamellae. Nevertheless, collagen lamellae interweave in the anterior flaps and stronger supramolecular organizations are formed, which significantly improve their tensile properties.

Collagen crosslinking of the posterior lamellae are not common. Nevertheless, it is noted that it could be used in vitro and for donor tissues. Furthermore, there are previous studies, which tried to combine crosslinking with other refractive surgical procedures. For example, laser-assisted in situ keratomileusis (LASIK) is a refractive laser surgery, in which the surgeon severs the anterior portion of the cornea in order to alter central corneal curvature. This procedure could reduce corneal biomechanical stability because it alters the microstructure of the tissue by cutting and ablation of collagen lamellae. One possible complication of LASIK is corneal ectasia [34]. It has been suggested to use collagen crosslinking treatment to increase the stiffness of the remaining cornea [35, 36]. The results presented here suggest that extra care should be taken in such approaches as the collagen crosslinking seems to have limited effect on posterior layers. Future studies could possibly introduce the thickness of residual stromal bed as the inclusion criteria for such therapeutic options. More importantly, many studies on different aspects of the collagen crosslinking treatment have been done using normal cornea; primarily from animals and to lesser amount from human donors. It is important to realize that the effect of this treatment procedure may be different on diseased tissue. It has been shown that keratoconus affects the corneal collagen microstructure and the proteoglycan content [37, 38]. The main difference between posterior and anterior layers are their ultrastructure. Here, an insignificant effect of corneal collagen crosslinking on posterior flaps was observed. Thus, we hypothesize that conclusions and protocol modifications that have been proposed using animal and/or healthy donor tissue should not be generalized and be used as a rational for future human studies in clinics. In other words, surgeons should be careful in practicing such ideas on their patients without any further in vitro studies on keratoconus corneas.

In summary, the present study provided enough evidence to conclude that corneal collagen crosslinking had an insignificant effect on biomechanical properties of posterior layers of the porcine cornea. Since these layers were directly crosslinked, this peculiar observation should not be due to insufficient riboflavin uptake. The microstructure of the collagen lamellae, and the specifications of collagen fibrils and proteoglycans are different in posterior and anterior regions. We are currently investigating in more details this problem in our laboratory and will present our findings in future publications.

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Conflict of Interest

None

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Figure Legends:

Figure 1. Schematic plot showing the anterior and posterior flaps. A DSAEK system was used to excise an anterior flap from the porcine cornea. The posterior flap was the remaining of the tissue after dissection of the anterior flap. From the flap, corneal strips were punched and were crosslinked using a custom made crosslinking device. The thickness of both anterior and posterior flaps were almost 400 microns.

Figure 2. Tensile stress-strain behavior of anterior and posterior flaps excised from porcine cornea. The anterior flaps showed a stiffer response than the posterior flaps. Furthermore, collagen crosslinking enhanced the biomechanical properties of the anterior flaps. However, it had no significant effect on the tensile behavior of posterior flaps.

Figure 3. Maximum tensile stress and tangent modulus of strips excised from the posterior and anterior region. A significant amount of stiffening was observed in anterior flaps ($p<0.05$) but posterior flaps were not stiffened by collagen crosslinking. Furthermore, anterior flaps showed a significantly stronger tensile properties than the posterior flaps ($p<0.05$).

Figure 4. Tensile stress-strain behavior of full thickness porcine corneas which were crosslinked from the anterior (top) or posterior (bottom) side. The collagen crosslinking therapy improved the tensile properties only when it was performed from the anterior (top) side.

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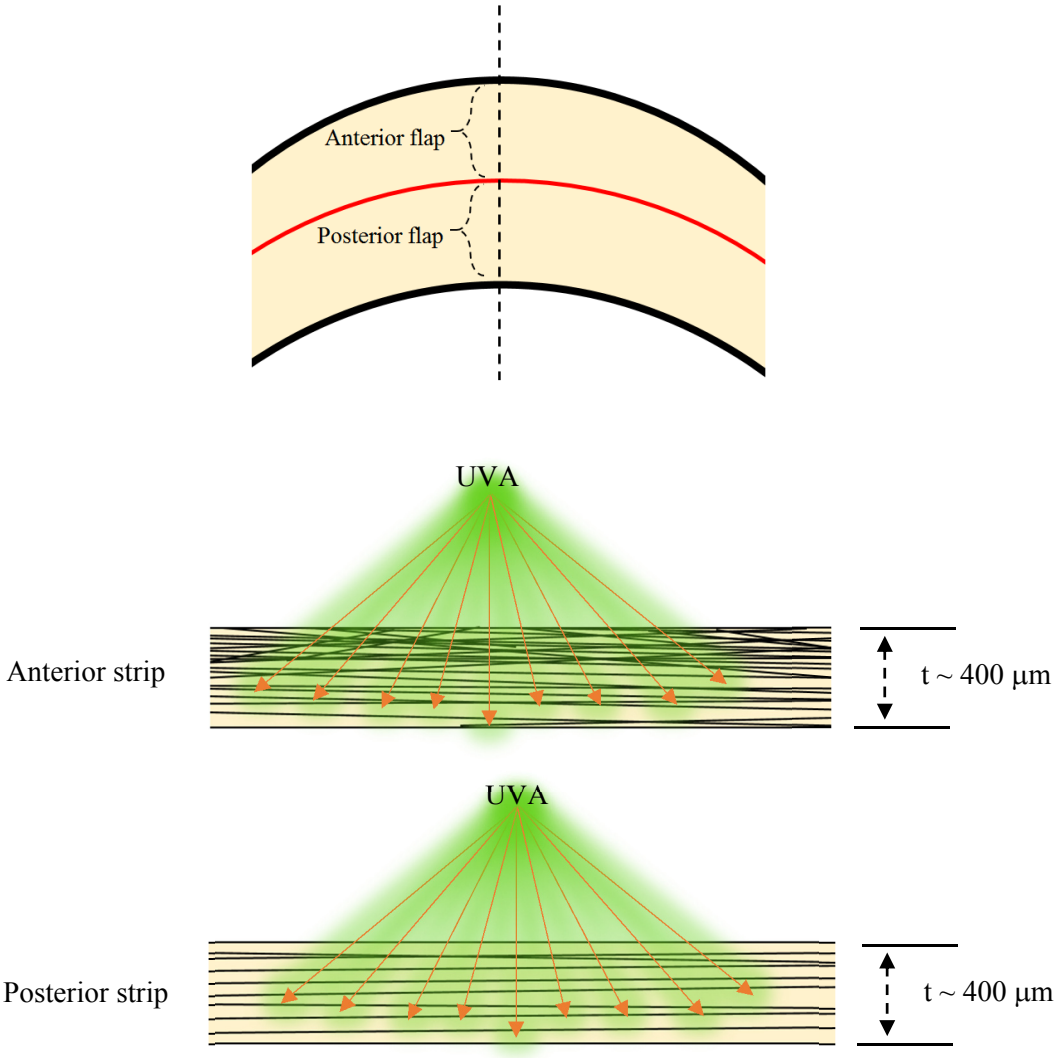

Figure 1 Schematic plot showing the anterior and posterior flaps. A DSAEK system was used to excise an anterior flap from the porcine cornea. The posterior flap was the remaining of the tissue after dissection of the anterior flap. From the flap, corneal strips were punched and were crosslinked using a custom made crosslinking device. The thickness of both anterior and posterior flaps were almost 400 microns.

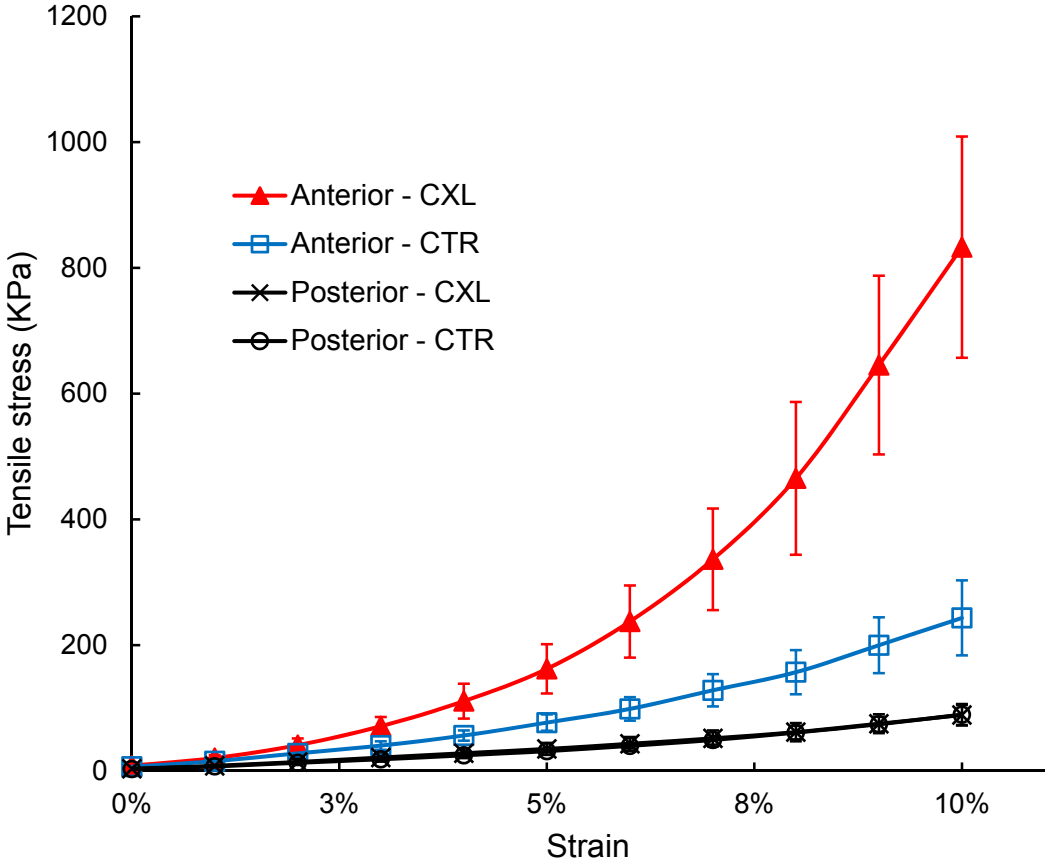

Figure 2 Tensile stress-strain behavior of anterior and posterior flaps excised from porcine cornea. The anterior flaps showed a stiffer response than the posterior flaps. Furthermore, collagen crosslinking enhanced the biomechanical properties of the anterior flaps. However, it had no significant effect on the tensile behavior of posterior flaps.

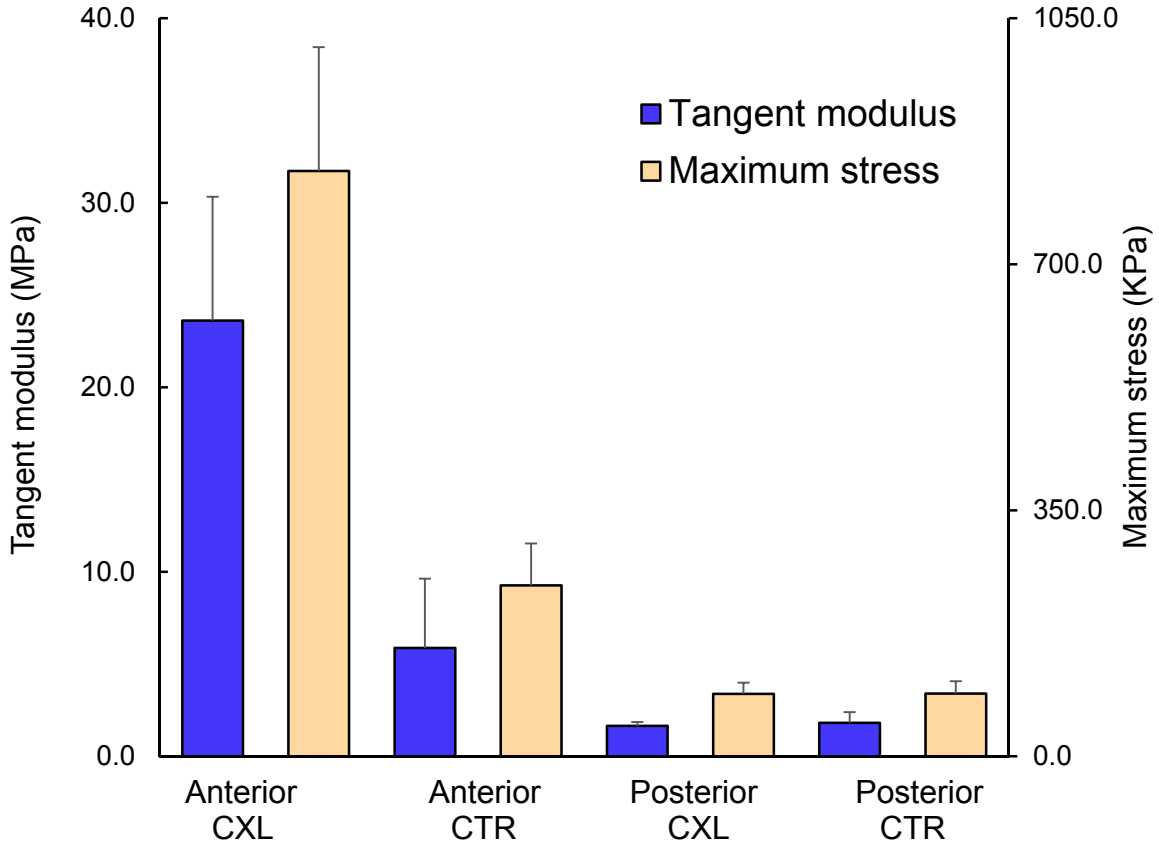

Figure 3 Maximum tensile stress and tangent modulus of strips excised from the posterior and anterior region. A significant amount of stiffening was observed in anterior flaps ($p<0.05$) but posterior flaps were not stiffened by collagen crosslinking. Furthermore, anterior flaps showed a significantly stronger tensile properties than the posterior flaps ($p<0.05$).

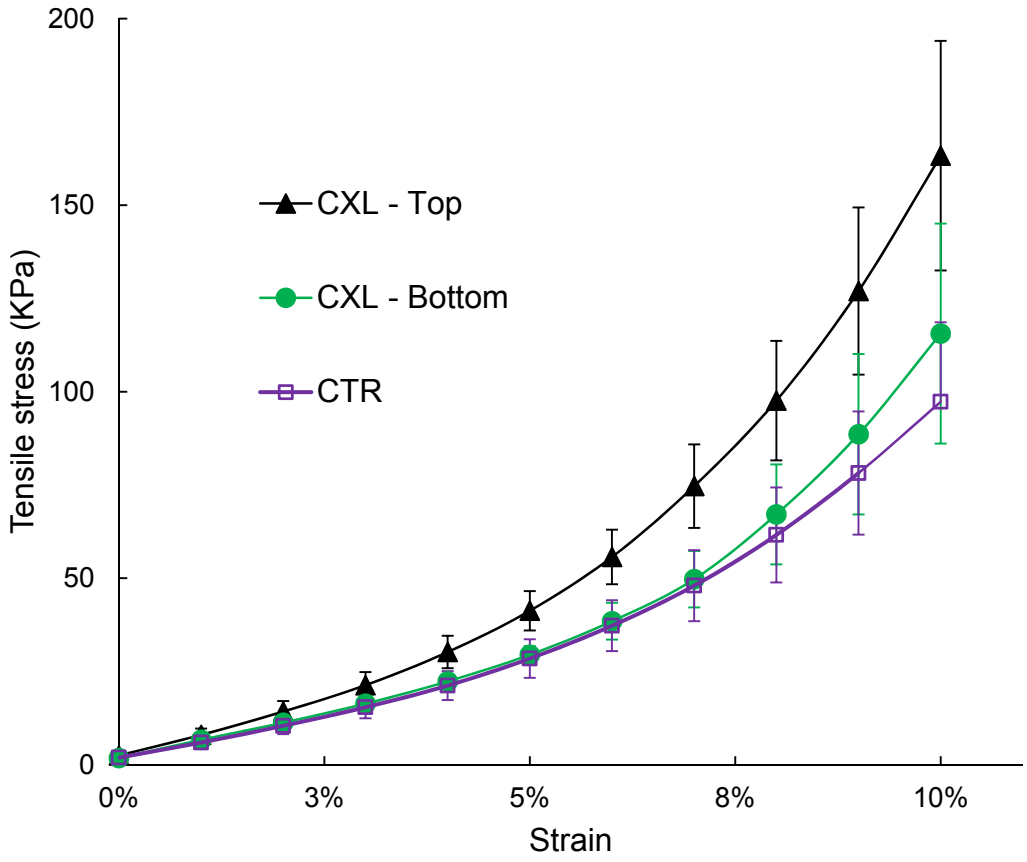

Figure 4 Tensile stress-strain behavior of full thickness porcine corneas which were crosslinked from the anterior (top) or posterior (bottom) side. The collagen crosslinking therapy improved the tensile properties only when it was performed from the anterior (top) side.