The impact of different rose bengal formulations on corneal thickness and the efficacy of rose bengal/green light cross-linking in the rabbit eye

Running head: Rb formulations affect CCT and RGX efficacy

Rongrong Gao MD PhD,[1#] Mengdi Yan MD,[1#] Ming Chen MD,[1] Sally Hayes PhD,[2] Keith M. Meek DSc PhD,[2,3] Huanhuan He MD,[1] Xueyang Chen MD,[1] Wenjin Xu MD,[1] Shixiang Yan MD,[1] Yuyan Huang MD,[1] Shengnan Ding MD,[1] Qinmei Wang MD,[1*] Junhua Li MD,[1*] Jinhai Huang MD PhD[1,3*]

[1] School of Ophthalmology and Optometry and Eye Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, China.

[2] Structural Biophysics Research Group, School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK.

[3] Eye Institute and Department of Ophthalmology, Eye & ENT Hospital, Fudan University; Key Laboratory of Myopia, Chinese Academy of Medical Sciences, Shanghai, China.

[#]These authors contributed equally.

***Corresponding authors: e-mail:** Qinmei Wang wqm6@mail.eye.ac.cn; Junhua Li 54064308 @qq.com; Jinhai Huang vip999vip@163.com

Mailing address:

Eye Hospital of Wenzhou Medical University, 270 West Xueyuan Road, Wenzhou, Zhejiang, China, 325027. Tel: 86-577-88068880, Fax: 86-577-88832083.

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ABSTRACT

Purpose: To examine central corneal thickness (CCT) changes during in vivo rose bengal-green light corneal cross-linking (RGX) and compare the cross-linking efficacy of different rose bengal (Rb) formulations.

Methods: After epithelium removal, the right eyes of rabbits were immersed in Rb solution for 2 or 20 minutes, then the Rb distribution in the corneal stroma was analyzed by confocal fluorescence detection. During the RGX process, the CCT was measured at 7 time points. The left eyes served as untreated control group. Corneal enzymatic resistance and corneal biomechanics were tested to compare the RGX efficacy.

Results: The Rb infiltration depths were about 100 μm and 200 μm for the 2-minute and the 20-minute groups, respectively. CCT increased significantly after infiltration, then decreased significantly in the first 200 seconds of irradiation and decreased slowly for the next 400 seconds. The CCT of the 20 min groups was significantly higher than that of the 2 min groups ($P < 0.0001$). All the RGX treatments improved the corneal enzymatic resistance and corneal biomechanics, with the effects being greater in the 20 min groups. The inclusion of 1.1% hydroxypropyl methylcellulose (HPMC) in the Rb formulation helped to maintain CCT during irradiation, whilst not affecting either the infiltration of Rb or the efficacy of RGX.

Conclusions: Within the range studied, RGX efficacy increase with infiltration time. The incorporation of a 20-minute infiltration of 0.1% Rb-1.1% HPMC into the RGX

procedure may further improve the safety of the treatment and its prospects for clinical use.

Keywords: corneal cross-linking, rose bengal, 532 nm green light, hydroxypropyl methylcellulose, central corneal thickness

Introduction

Keratoconus is a progressive corneal degenerative disease, characterized by corneal thinning, irregular astigmatism and secondary visual impairment.[1] Corneal cross-linking is the main treatment to enhance the biomechanical properties of the cornea and delay the progress of keratoconus.[2, 3]

The standard corneal cross-linking protocol (UVX), often referred to as the Dresden protocol, involves the use of riboflavin and ultraviolet light, and requires a central corneal thickness (CCT) of no less than 400 μm after de-epithelialization to keep the irradiation dose within the safe range of the corneal endothelium.[4] However, it is sometimes difficult to achieve and maintain the required thickness throughout the UVX process, and the CCT of many patients before the operation is less than 400 μm.[5] Numerous clinical and laboratory studies have examined the efficacy of different riboflavin (Rf) formulations on CCT during UVX, and shown that variations in the Rf carrier solution can lead to considerable variations in the final CCT.[6-9]

Rose bengal-green light corneal cross-linking (RGX) is a promising treatment for thin corneas due to the shallow infiltration of rose bengal (Rb) in the corneal stroma.[10-14] Since the irradiance and total energy of light is much larger with RGX than UVX (0.25 W/cm^2 to 0.4 W/cm^2 in RGX vs. 3 mW/cm^2 in UVX), we hypothesized that water evaporation during light exposure might lead to a decrease in the CCT, thus affecting the safety of endothelial cells. Although RGX performed on laser-made 250 μm thick rabbit corneas at an illumination intensity of 0.4 W/cm^2 for 250s (100 J/cm^2) has been shown to be safe[15], maintaining a suitable CCT during surgery should further improve the safety of the technique, making it suitable for more patients. However, unlike UVX for which a variety of commercial Rf formulations have been developed to enable the customization of treatments, there is a lack of commercial Rb formulations and studies

to date have been limited to the use of a Rb formulation comprising 0.1% Rb in phosphate buffered saline (PBS). To our knowledge, the efficacy of this Rb formulation on CCT has not yet been reported.

The Rf formulation used in the Dresden UVX protocol comprises 0.1% riboflavin in 20% dextran T500. The dextran increases the solution viscosity and has good film-forming performance with an average film rupture time of 22 minutes.[16] However, due its strong hydrophilic hydroxyl groups and hyperosmolarity, its application can cause corneal dehydration and result in a significant decrease in CCT.[6] The use of hydroxypropyl methylcellulose (HPMC) as an alternative Rf carrier solution, has some advantages over dextran in that it offers a longer average film rupture time of 32 minutes and it does not cause significant corneal dehydration or tissue thinning.[6, 17, 18] However, it is still controversial which is more effective in UVX, the use of Rf solutions containing HPMC or those containing dextran. Based on a retrospective analysis of 24-month follow-up data from 33 patients that underwent UVX with either a HPMC Rf formulation or a dextran Rf formulation, Rapuano et al. concluded that the dextran Rf formulation may result in significantly better visual acuity compared to the isotonic HPMC Rf formulation.[19] Contrary to this, Thorsrud et. al's study of 40 patients at 2-years follow-up showed the opposite, i.e. that UVX with Rf solutions containing HPMC had a better efficacy on visual outcomes than UVX with Rf solutions containing dextran.[7] In light of the above, we postulate that HPMC may be appropriate for maintaining the CCT in the process of RGX, but its efficacy on RGX needs to be explored.

The osmotic pressure of the photosensitizer formulation is another important factor that affects CCT. In some cases, hypotonic Rf formulations have been used to swell very thin corneas to ensure that they achieve the minimum thickness required for UVX

treatment but this efficacy can be transient and unstable due to the endothelial cell function and the evaporation of corneal surface water.[17, 18, 20] The Rb formulation used in previously published RGX studies was 0.1% Rb in PBS and the effect of other Rb formulations on CCT is as yet unknown.

The present study aims to explore the CCT changes in rabbit corneas during in vivo RGX. We also examine the surgical efficacy of different Rb formulations that vary in terms of their carrier solution, concentration and infiltration time.

Materials and Methods

Materials

All chemicals used in the preparation of the different Rb formulations (Table 1), were purchased from Sigma-Aldrich, including Rb, dextran (»500 kDa) and HPMC. The concentration of Rb in all of the prepared formulations was 0.1% weight/volume. 0.2% type II collagenase was also purchased from Sigma-Aldrich, prepared as a 0.2% weight/volume solution in PBS and kept at -4 °C.

Experimental Animals

Clean grade male Japanese white rabbits (2.5-3 kg) were supplied by the experimental animal center of Wenzhou Medical University. No abnormal anterior segment was observed by slit lamp. The feeding environment was good, the food and water were supplemented regularly. This experiment was granted by the animal ethics committee of Wenzhou Medical University (NO. wydw 2021-0056). The welfare and use of the experimental animals complied with the ARRIVE guidelines and were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated

guidelines, EU Directive 2010/63/EU for animal experiments. After treatment, rabbits were euthanized by inhaling excessive carbon dioxide.

Animal grouping

Rabbits were randomly divided into 12 treatment groups, in which the right eye of each animal was treated with a different combination of the formula of the Rb solution and infiltration time, and the left eye was de-epithelialized as the untreated control group (Table 1).

Rb infiltration test

Rabbits were anesthetized by intramuscular injection. After topical ocular surficial anesthesia, the central 8 mm diameter corneal epithelium was removed, and the corneal surface of each group was completely infiltrated by the corresponding Rb formulation via a corneal well for either 2 or 20 minutes. After euthanasia, 5 mm diameter central corneal buttons were trephined and 10 μm frozen sections were cut. Rb fluorescence of corneal sections were photographed using a Zeiss 710 confocal microscope with an excitation wavelength of 543 nm and an emission wavelength of 600 nm. ImageJ v1.51j8 software was used to analyze the Rb fluorescence (n=4).

RGX and CCT measurement

After Rb infiltration, the other experimental corneas were irradiated immediately with 0.25 W/cm^2 green light for 600 sec. During this time, a 30 second re-application of the

respective Rb formulation was performed at 200 sec and 400 sec of irradiation, and the cornea was rinsed with PBS at the end of the irradiation procedure. CCTs were measured with an ultrasound pachymeter (USP; SP-3000, Tomey Corp., Nagoya, Japan) at the following 7 time points: before de-epithelialization, after de-epithelialization, after infiltration, after irradiation for 200 sec, 400 sec and 600 sec, and after rinsing. All CCT measurements were performed 5 times by one well experienced operator and the average value recorded. After RGX, the rabbits were euthanized and used for further experiments as follows.

Corneal enzymatic resistance test

After euthanasia, an 8 mm diameter central corneal button was trephined from each eye and digested in 0.2% type II collagenase at a constant temperature of 37 °C. The undigested corneal buttons were photographed every 2 hours until complete digestion. The sample areas were calculated using ImageJ software, and area versus time curves were drawn (n=4).

Corneal biomechanics test

After animal euthanasia, the central vertical 3mm width corneal strips with 3mm sclera were cut with a double-edged knife and placed in a universal testing machine (Model 3343, Instron Corp., Canton, Mass., USA). The strips' initial lengths were set as 10 mm and the extension rate was set as 2 mm/min. The strips were stretched to a displacement

of 1 mm, then returned to displacement of 0, and this was cycled three times with a recovery of 30 sec between cycles. Finally, the strips were stretched to 20% deformation. The stress-strain curves were drawn, and the slopes of the curves (i.e. the Young's modulus) at different strains were calculated by the instrument's software (n=4).

Statistical analysis

The data and statistical charts were processed by GraphPad Prism v8.2.1 software (San Diego, USA). Single factor analysis of variance and multi factor analysis of variance were used. $P < 0.05$ indicated statistical significance.

Results

Rb infiltration test

The presence of HPMC did not affect the infiltration of Rb. The infiltration depths were about 120 μm in the 2 min groups and 200 μm in the 20 min groups. Both the infiltration depth and the areas under the fluorescence versus depth curves (AUCs) increased significantly with the extension of infiltration time (Figure 1 and Table 2). The groups containing dextran demonstrated the minimal AUCs, with the values being about 10% that of the other treatment groups with the same infiltration time, and thus were not included in the follow-up experiments. There was no significant difference of the AUCs among the other groups with the same infiltration time (Figure 1).

CCT changes during RGX

The CCTs (recorded at specific time points during each treatment) minus the CCTs after de-epithelialization were recorded as △CCTs. Table 3 and Table 4 show the CCTs and the △CCTs of each group at different time points. The average initial CCT (before de-epithelialization) ranged from 364 μm to 372 μm (Table 3), and the average corneal epithelial thickness ranged from 45 μm to 55 μm among groups (Table 4). There was no significant difference among the groups (P>0.05).

As shown by Figure 2, the overall trend in the CCT variation during the RGX process was that the CCTs increased significantly in all groups after infiltration (about 70 μm in the 2 min groups and 170 μm in the 20 min groups) with the exception of the hypotonic 0.1% Rb groups which showed only a slight increase in CCT (about 30 μm) (Figure 2 A1, A2, B1). The CCT of all the groups decreased significantly during the first 200 sec of irradiation (Figure 2 A1, A2, B2), and then decreased slowly during the last 400 sec of irradiation (Figure 2 A1, A2, B3, B4). The groups with HPMC concentration of 1.1% and 1.7% maintained larger CCTs during irradiation than groups with other Rb formulations (P < 0.05) (Figure 2 A1, A2, B2-4). In all treatment groups, the CCTs increased after rinsing (Figure 2 A1, A2).

There was no significant CCT difference between the 2 min and 20 min infiltration protocol of 0.1% Rb-water (P > 0.05), and the CCTs of the two 0.1% Rb-water groups were both lower than that of other groups during RGX. Except for these two groups,

the CCTs of other formulations in the 20 min groups were significantly greater than 2 min groups during RGX ($P < 0.05$) (Figure 2).

Corneal enzymatic resistance test

Figure 3 shows groups of photos taken every two hours. The untreated corneas were digested most rapidly, being completely digested within 6 to 8 hours. The digestion times in all experimental groups were longer than the untreated control group ($P < 0.05$). The average digestion time varied from 11.5 to 14 hours in the 2 min groups, and 17 to 19.5 hours in the 20 min groups. Overall, the digestion time of the 20 min groups were about 5 to 6 hours longer than the 2 min groups with the same formulation ($P < 0.05$, Table 5). There was no significant difference in the digestion time among experimental groups with the same infiltration time. Separation of the anterior and posterior stroma during the enzyme digestion was observed between 6 to 8 hours in some RGX-treated corneas (Figure 3 A2). Their anterior stroma was able to be maintained in collagenase solution for a long time, while the posterior stroma was completely digested at a rapid rate once separated.

Corneal biomechanics test

According to the above results, cross-linking in the 0.1% Rb-1.1% HPMC 20 min group showed a good combination of a thick CCT and excellent enzyme resistance, so it was chosen for the corneal biomechanics test. The 0.1% Rb-PBS group and the untreated

group were also included as a routine control and a negative control respectively. Although hypotonic groups resulted in significant improvements in the resistance of the cornea to enzyme digestion, they were abandoned because of the steep decline of CCT during the irradiation procedure. Table 6 and Figure 4 show the Young's modulus of corneal strips at different strains. At 10% strain, the untreated group had the smallest average Young's modulus with a value of 18.95 ± 2.12 MPa. The 0.1% Rb-PBS 2 min group, 0.1% Rb-PBS 20 min group and 0.1% Rb-1.1% HPMC 20 min groups' Young's moduli were 32.55 ± 2.31 MPa, 39.80 ± 1.53 MPa and 38.72 ± 4.50 MPa, respectively, i.e. 1.72, 2.10 and 2.04 fold the value of the untreated group, respectively ($P < 0.05$). 0.1% Rb-PBS 2 min was significantly lower than that of 0.1% Rb-PBS 20 min and 0.1% Rb-1.1% HPMC 20 min ($P < 0.05$). There was no significant difference in Young's modulus between the last two groups ($P > 0.05$).

Discussion

UVX cross-links the anterior 250 to 300 μm of the corneal stroma, and increases corneal stiffness by about 3-fold.[21] However, many keratoconus patients with thin corneas do not meet the traditional UVX requirement that the de-epithelialized CCT should be greater than 400 μm to ensure that the UVA irradiance of endothelial cells remains lower than the toxicity threshold of 0.35 mW/cm^2.[22]

In a small pilot study, Mark et al.[9] compared UVX with different formulations of Rf which varied in their type and concentration of carrier solution. They found that the mean post-treatment CCTs were 1.72, 1.83 and 1.70 folds of the preoperative values in Rf formulations which contained 0.5%, 1.0% and 1.7% HPMC respectively, while CCT

reduced to 0.80 of its initial value when a Rf-10% dextran formulation was used. Thorsrud et al.[7] found that although the maximum corneal curvature (K_{max}) and best-corrected visual acuity (BCVA) of patients treated with Rf-dextran remained stable at 2-years follow-up, those treated with Rf-HPMC showed significant improvements in both parameters, suggesting that UVX using Rf-HPMC can produce a deeper stromal effect. Hammer et al.[23] found in rabbits that the corneal Rf concentration of the Rf-HPMC groups was 4 to 18 times higher than that of Rf-dextran groups. Similar results were obtained by Ehmke et al.[24] in porcine corneas.

Rb is a halogenated xanthene dye that is often used as a diagnostic agent for corneal surface damage and is approved by FDA.[25] Both Rb and Rf have been used as oxidative photosensitizers for photosensitized protein cross-linking. Although their photophysical properties are similar, Rb associates tightly with collagen whereas Rf diffuses freely,[25, 26] and the effect of formulation components on the permeation of Rb may be different from that of Rf. We observed that the Rb infiltration depths were about 120 μm after a 2 min infiltration and 200 μm after a 20 min infiltration (Table 2). The depth of the 2 min group was 20μm deeper than a previous report,[10] and the depth of the 20 min group also differed from Wang et. al who found that most Rb was localized within the superficial 120 μm of the rabbit corneal stroma.[15] This discrepancy is likely due to differences in the application method, as Wang et. al applied 0.1% Rb at 5 min intervals over a period of 20-minutes, and then allowed the tissue to absorb it for a further 10 minutes in the dark, while in this study Rb was applied via a corneal well to ensure continuous soaking of the corneal surface for 20 minutes without further absorption. Since the Rb formulation can easily flow away, the more continuous contact is conducive to its penetration into the cornea. We confirmed that the presence of HPMC did not affect the infiltration of Rb, while the groups that contained dextran

demonstrated the shallowest penetration depth (Figure 1). We speculate that Rb may bind to dextran physically or chemically, thus hindering its penetration into the cornea. The specific mechanism needs to be verified by more studies in the future.

The green light irradiation energy used in RGX (150 J/cm^2 in the current study) is much higher than the energy of the ultraviolet rays used in UVX. It was reported that the corneal surface temperature increased by less than 8 ℃ during the irradiation period[27]. Water evaporation may lead to a significant reduction of CCT during the process of irradiation, especially in the first 200 seconds. The CCTs of the 0.1% Rb-PBS 2 min group and the 20 min group were respectively (40 ± 19) μm and (74 ± 13) μm thinner after irradiation for 600 sec than after de-epithelialization (Figure 2). The significant reduction may lead to potential safety hazards associated with RGX. HPMC is a non-ionic cellulose polymer often used as a lubricant in ophthalmology. Wollensak et al.[16] measured the thickness of the Rf film formed by different Rf formulations on the corneal surface, and found that the thicknesses were 300 μm, 70 μm and 40 μm for Rf-HPMC, Rf-dextran and Rf-saline (Medio-Cross hypotonic solution)solutions, respectively. The good film-forming property of HPMC can prevent water evaporation from the corneal tissue and the consequent reduction of CCT during irradiation. The results of our study revealed that Rb-HPMC produced the same RGX efficacy as 0.1% Rb-PBS formulation whilst also maintaining the thickness of the cornea during irradiation. These findings indicate that the use of Rb-HPMC may be seen as a promising modification to the RGX treatment to improve patient safety. Another important finding of this study was that the groups treated with a hypotonic Rb formulation had CCTs significantly lower than all other groups during the whole infiltration and irradiation process; the difference was as high as 110 μm after irradiation for 600 sec, thus it is not recommended for RGX.

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Cherfan et al.[10] showed that an RGX treatment (0.1% Rb-PBS application for 2 min, 150 J/cm^2) increased the corneal Young's modulus 4.4 fold compared with the untreated group (16.3± 4.08 MPa vs. 3.72 6 ± 1.69 MPa, $P < 0.05$) in fresh young rabbit eyes. Due to factors such as corneal edema in vitro, the stiffness of their in vitro untreated group was found to be lower than that of the in vivo untreated corneas. Zhu et al.[27] found RGX in vivo using the same protocol increased the Young's modulus of rabbit corneas by a factor of 1.72 on day 1 compared with control untreated corneas (10.9 ± 3.37 N/mm^2 vs. 6.33 ± 1.38 N/mm^2, $P < 0.05$). We carried out the biomechanical testing immediately after RGX, and the increase in Young's modulus was also 1.72-fold in the 0.1% Rb-PBS 2 min group, consistent with the Zhu et al. study. Besides, the current study showed that the 0.1% Rb-PBS 20 min group and 0.1% Rb-1.1% HPMC 20 min groups improved the corneal stiffness to 2.10 and 2.04 folds of the untreated group respectively at 10% strain ($P < 0.05$), and their slight difference was not statistically significant (Table 6, Figure 4). Our findings suggest that the RGX efficacy of Rb soaking for 20 minutes was better than for 2 minutes, and the addition of HPMC did not affect the outcome of surgery.

Appropriate intraoperative corneal thickness needs to consider the balance between safety and efficacy of photosensitized protein cross-linking. Some studies on UVX suggested that an increase in corneal thickness may deteriorate the cross-linking efficacy since the percentage of the cross-linked cornea was decreased.[28, 29] However, the HPMC maintained the CCT (even thicker than before cross-linking) without blocking Rb penetration or weakening the efficacy of RGX. We speculate that the reasons may be the high penetration of green light and/or the collagen binding properties of Rb. The cross-linking was located in the anterior part of the cornea, confirmed by the fact that the un-cross-linked posterior stroma was easily digested,

while the corneal thickening may mainly occur in the middle and posterior part of the cornea.

Unexpectedly, the efficacy of HPMC in maintaining CCT did not increase with the increase of its concentration, the maximum efficacy was observed at 1.1% concentration. Furthermore, increasing the HPMC concentration to 1.7% decreased the Rb infiltration depth and the resulted in a lower CCT during irradiation than that achieved with the 1.1% concentration. Similar results were found in UVX by Mark et. al.[9] who increased the HPMC concentration in the Rf drops from 0.5% to 1.0% and 1.7%, with final CCTs of 172%, 183% and 170% in the patient cornea. What is more, an exorbitant increase in HPMC concentration raises the viscosity of the formulation, thus reducing its practicality.

There were some limitations in the present study. First of all, previous studies of rabbit corneas at 1 and 28 days after RGX have shown that the corneal stiffness continues to increase after treatment,[27] but here we only evaluated the immediate efficacy after RGX without follow-up. Secondly, the Rb infiltration times examined were limited to just 2 and 20-minutes. Although a 20-minute infiltration time of Rb-HPMC resulted in the greatest RGX efficacy, further studies are warranted to determine the optimal infiltration time in terms of maximizing the RGX efficacy and minimizing the patient treatment time.

Conclusion

In RGX, the CCT increased after infiltration but decreased significantly during irradiation, especially over the first 200 sec. The addition of HPMC in the Rb formulation slowed down the reduction of CCT during RGX without affecting either the infiltration of Rb into the cornea or the cross-linking efficacy. 0.1% Rb-1.1%

363 HPMC infiltration for 20 minutes RGX is likely to have considerable potential for
364 future clinical applications.

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Figures

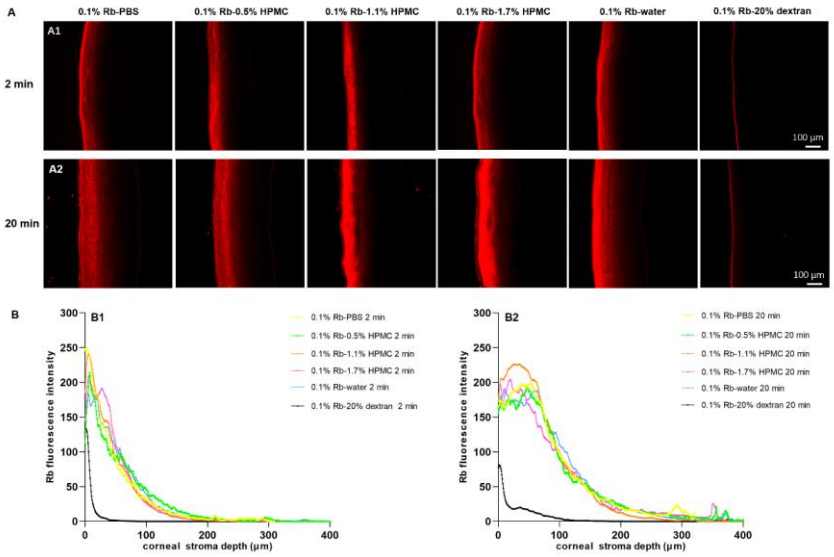
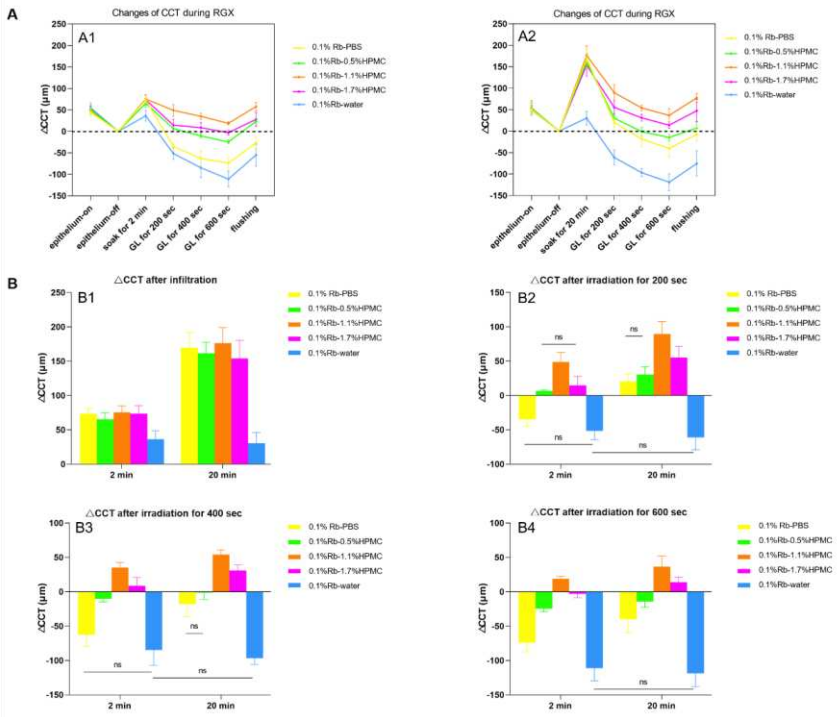


Figure 1. Rb fluorescence distribution in the corneal stroma (n = 4). A) Fluorescence photos of corneal sections. Rb fluorescence was red, magnification: 10X, scale: 100 μm. B) Rb fluorescence distribution curves of different Rb formulations with the same infiltration time of 2min (B1) and 20min (B2)



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465 **Figure 2.** Changes of CCT during RGX (n = 5). A) △CCT at different time points

466 during RGX in groups with infiltration time of 2 minutes (A1) and 20 minutes (A2). B)

467 The average △CCT of each group after infiltration (B1), after irradiation for 200sec

468 (B2), 400sec (B3) and 600sec (B4).

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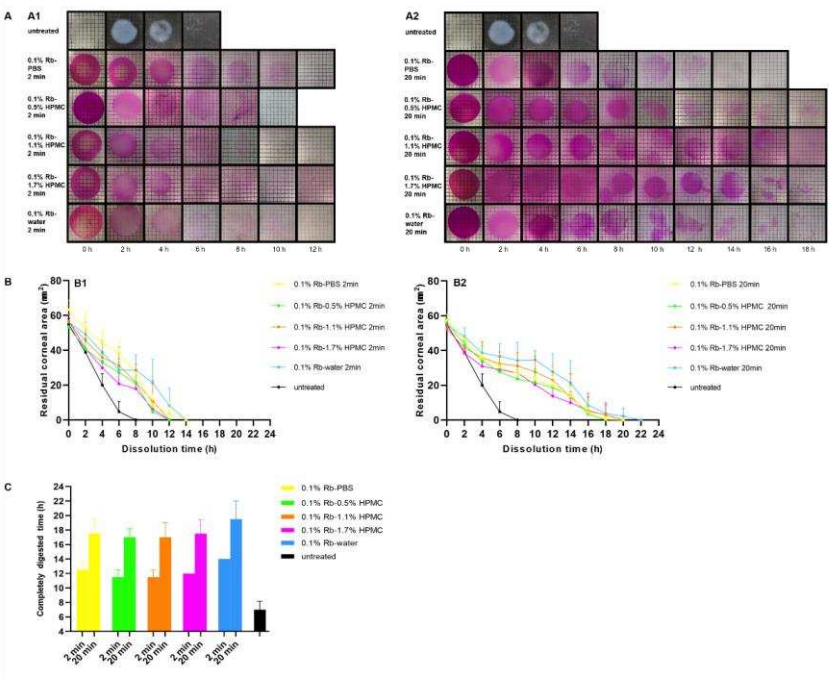
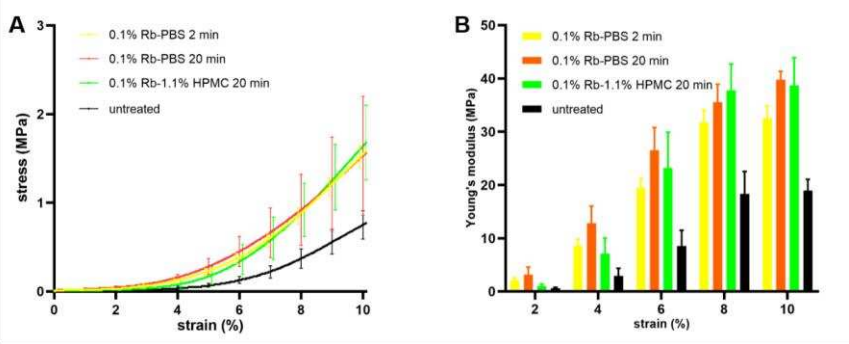


Figure 3. Corneal enzymatic resistance (n = 4). A) Photos of corneal buttons at 2-hour intervals. (A1) Photos of 2 min groups and the untreated control group; (A2) Photos of 20 min groups and the untreated control group. B) Average residual corneal button area for each treatment group decreased with time. (B1) Corneal digestion curves of 2 min groups and the untreated group; (B2) Corneal digestion curves of 20 min groups and the untreated group. C) Comparison of times required for complete digestion (mean ± SD).



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485 **Figure 4.** Corneal biomechanics (n = 4). A) Stress-strain curves of corneas treated with

486 RGX. B) The Young's modulus of corneal strips at different strains.

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