·Basic Research·

Riboflavin concentration in corneal stroma after intracameral injection

Na Li[1,2], *Xiu-Jun Peng*[2], *Zheng-Jun Fan*[2], *Xu Pang*[2], *Yu Xia*[2], *Teng-Fei Wu*[2]

[1]Chinese PLA Medical School & Chinese PLA General Hospital, Beijing 100853, China
[2]Department of Ophthalmology, Navy General Hospital of Chinese PLA, Beijing 100048, China
Correspondence to: Xiu-Jun Peng. Department of Ophthalmology, Navy General Hospital of Chinese PLA, 6 Fu-Cheng Rd, Beijing 100048, China. pxj1@vip.sina.com
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Abstract

• AIM: To evaluate the enrichment of riboflavin in the corneal stroma after intracameral injection to research the barrier ability of the corneal endothelium to riboflavin *in vivo*.

• METHODS: The right eyes of 30 New Zealand white rabbits were divided into three groups. Different concentrations riboflavin-balanced salt solutions (BSS) were injected into the anterior chamber (10 with 0.5%, 10 with 1%, and 10 with 2%). Eight corneal buttons of 8.5 mm in diameter from each group were dissected at 30min after injection and the riboflavin concentrations in the corneal stroma were determined using high-performance liquid chromatography (HPLC) after removing the epithelium and endothelium. The other two rabbits in every group were observed for 24h and sacrificed. As a comparison, the riboflavin concentrations from 16 corneal stromal samples were determined using HPLC after instillation of 0.1% riboflavin-BSS solution for 30min on the corneal surface (8 without epithelium and 8 with intact epithelium).

• RESULTS: The mean riboflavin concentrations were 11.19, 18.97, 25.08, 20.18, and 1.13 μg/g for 0.5%, 1%, 2%, de-epithelialzed samples, and the transepithelial groups, respectively. The color change of the corneal stroma and the HPLC results showed that enrichment with riboflavin similar to classical de-epithelialized corneal collagen crosslinking (CXL) could be achieved by intracameral 1% riboflavin-BSS solution after 30min; the effect appeared to be continuous for at least 30min.

• CONCLUSION: Riboflavin can effectively penetrate the corneal stroma through the endothelium after an intracameral injection *in vivo*, so it could be an enhancing method that could improve the corneal riboflavin concentration in transepithelial CXL.

• KEYWORDS: riboflavin; cornea; intracameral injection; high-performance liquid chromatography; endothelium
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INTRODUCTION

Riboflavin/ultraviolet-A (UVA)-induced corneal collagen crosslinking (CXL) is the first treatment available for stabilization of the keratoconic process while aiming at its pathogenetic cause; this process was first used in the 1990s at Dresden University, Germany [1,2]. The photosensitizer riboflavin that has reached the corneal stroma is irradiated by UVA; this generates reactive oxygen species and induces chemical covalent bonds that bridge the amino groups of collagen fibrils. Consequently, it leads to an increase in the biomechanical stabilization and stiffness of the cornea [3,4]. In classical CXL, removing the corneal epithelium is required for adequate penetration of riboflavin (molecular weight 376.36 g/mol) into the corneal stroma [5-7]. But the possible risks of corneal infections, ulcers, postoperative pain, and long recovery has made research about transepithelial CXL a hot topic in ophthalmology. A sufficient concentration of riboflavin in the corneal stroma is vital for efficacious and safe performance of transepithelial CXL[8-11].

Recently, different methods of CXL using penetration enhancers [12-15] or iontoforesis have been promising [16-19]; however, these formats need special riboflavin solutions containing penetration enhancers or the use of iontoforesis instruments, and the accumulation of riboflavin is not enough. Direct introduction by the pocket technique or needle technique [20-22] is indeed efficacious but the process is obviously complicated. In 2011, Bottós *et al* [9] studied the immunofluorescence microscopy results of enucleated porcine eyes after a 0.1% riboflavin-20% dextran solution was injected into the anterior chamber and UVA irradiation was applied.

But the actual riboflavin concentration in the corneal stroma after intracameral injection is unknown; here we assessed the penetration levels of riboflavin through the endothelium after intracameral injection through an intact epithelium *in vivo*

to explore the clinical apllication of this intensive transepithelial CXL method. The method is simple but invasive. Considering the importance of a sterile environment and the stability of intraocular pressure for intracameral injection, different concentrations of riboflavin-balanced salt solutions (BSS) 0.1 mL were used in animals in this study. The concentrations of stromal riboflavin were determined by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Study Design Forty-six right eyes of 46 New Zealand white rabbits without eye disease (without regard to gender) that weighed 2.0-2.5 kg were used in the experiments. Thirty rabbits were divided into three treatment groups and different concentrations of riboflavin-BSS solutions were injected into the anterior chamber (10 with 0.5%, 10 with 1%, and 10 with 2%). Eight corneal buttons with 8.5 mm diameter in each group were dissected after 30min and the riboflavin concentrations in the corneal stroma were determined by HPLC after removing the epithelium and endothelium. The other two rabbits in each group were observed for 24h and sacrificed to roughly assess the metabolism of riboflavin. In a control group, 16 corneal stromal riboflavin concentrations were determined by HPLC after instillation of 0.1% riboflavin-BSS solution on the corneal surface (8 without epithelium and 8 with intact epithelium). All animal procedures were approved by the ethics committee and conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Treatment with Intracameral Riboflavin General anesthesia was induced with an intramuscular injection of a mixture of xylazine hydrochloride (1.5 mL) and ketamine (2 mL) using a dosage of 0.3 mL/kg. The rabbits were placed on their left side with the right eye upward; the right eye was held open with a blepharostat. Different concentrations of riboflavin-BSS solution (riboflavin-5-phosphate, BSS) 0.1 mL were drawn into a 1 mL scale syringe and injected into the anterior chamber through the corneal limbus parallel with the surface of the iris. A central corneal button with 8.5 mm diameter was dissected using a trephine and microscissors 30min after injection. The corneal epithelium and endothelium were removed with a spatula; the corneal stromal buttons were washed with 10 mL BSS, and placed in sterile screw-capped histology containers with aluminium foil to shield from light at -80℃ for HPLC examination. The rabbits were sacrificed postoperatively.

Treatment of Controls De-epithelialized instillation controls: after general anesthesia, rabbits were placed with the right eye upward and held open using a blepharostat; the corneal epithelium was debrided with a spatula. After the 0.1% riboflavin-BSS solution had been instilled for 30min every 3min, the corneal buttons were dissected. The endothelium was removed and the specimens were washed by BSS and stored.

Transepithelial instillation controls: after general anesthesia, rabbits were placed with the right eye upward and held open using a blepharostat. After 0.1% riboflavin-BSS solution instilled for 30min every 3min, the corneal buttons were dissected. The epithelium and endothelium were removed and the specimens were washed by BSS and stored.

Preparation of Corneal Specimens The corneal stromal buttons were weighed after defrosting and homogenized in a potassium dihydrogen phosphate buffer (PH 7.4) at 0℃. We mixed 1 mL of homogenate with 1 mL of magnesium acetate solution (15 mmol/L) and incubated the mixture at 65℃ for 15min. After the addition of 0.5 mL of trichloroacetic acid (10%) to the precipitated proteins and 10min of centrifugation (3200 r/min, 4℃), the supernatant was preserved. The pellet was mixed with 1 mL of magnesium acetate solution (15 mmol/L) and centrifuged again. The two supernatants were added together and infused into a C18-SPE cartridge previously activated with 2 mL methanol, then 1 mL purified water was used to wash the cartridge after the riboflavin absorption. After the last cartridge was washed with methanol and the extraction solution was collected, the solution was diluted 10 times for the HPLC examination.

High-performance Liquid Chromatography Analysis of Riboflavin The HPLC system (Hitachi L-2000) was equipped with a fluorescence detector; the chromatograph conditions for the riboflavin analysis included a chromatographic column (SinaChrom ODS-BP 5 μm, 250×4.6 -mm^2 I.D., Elite), the mobile phase (methanol-purified water v:v=40:60), the pH (7.0), the flow rate (1 mL/min), and the column temperature (40℃). The excitation and emission wavelengths of the spectrofluorometer were 425 nm and 525 nm, respectively. Diluted solution was determined by external standard method. The detection limit of riboflavin was 2×10^{-8} mg/mL. The concentration of the diluted solution was analyzed using calibration curves and the riboflavin peak area. The riboflavin concentration of the undiluted solution (the concentration of the diluted solution multiplied by diluted times) multiplied by the volume (methanol volume washed during the last step) equaled the riboflavin content. The riboflavin concentration in the corneal stroma was the content of riboflavin divided by the weighted mass of the stromal button.

Photographs of Eye Anterior Segments After general anesthesia, the degree of yellow-staining in the cornea and the anterior chamber inflammation reaction were observed by photograph. All specimens utilizing riboflavin in the experiments needed be preserved and shielded from the light.

Statistical Analysis Student's t-tests were used to compare the riboflavin concentrations values. Results with a P value less than 0.01 were considered statistically significant. Data analyses were performed using SPSS 16.0 software.

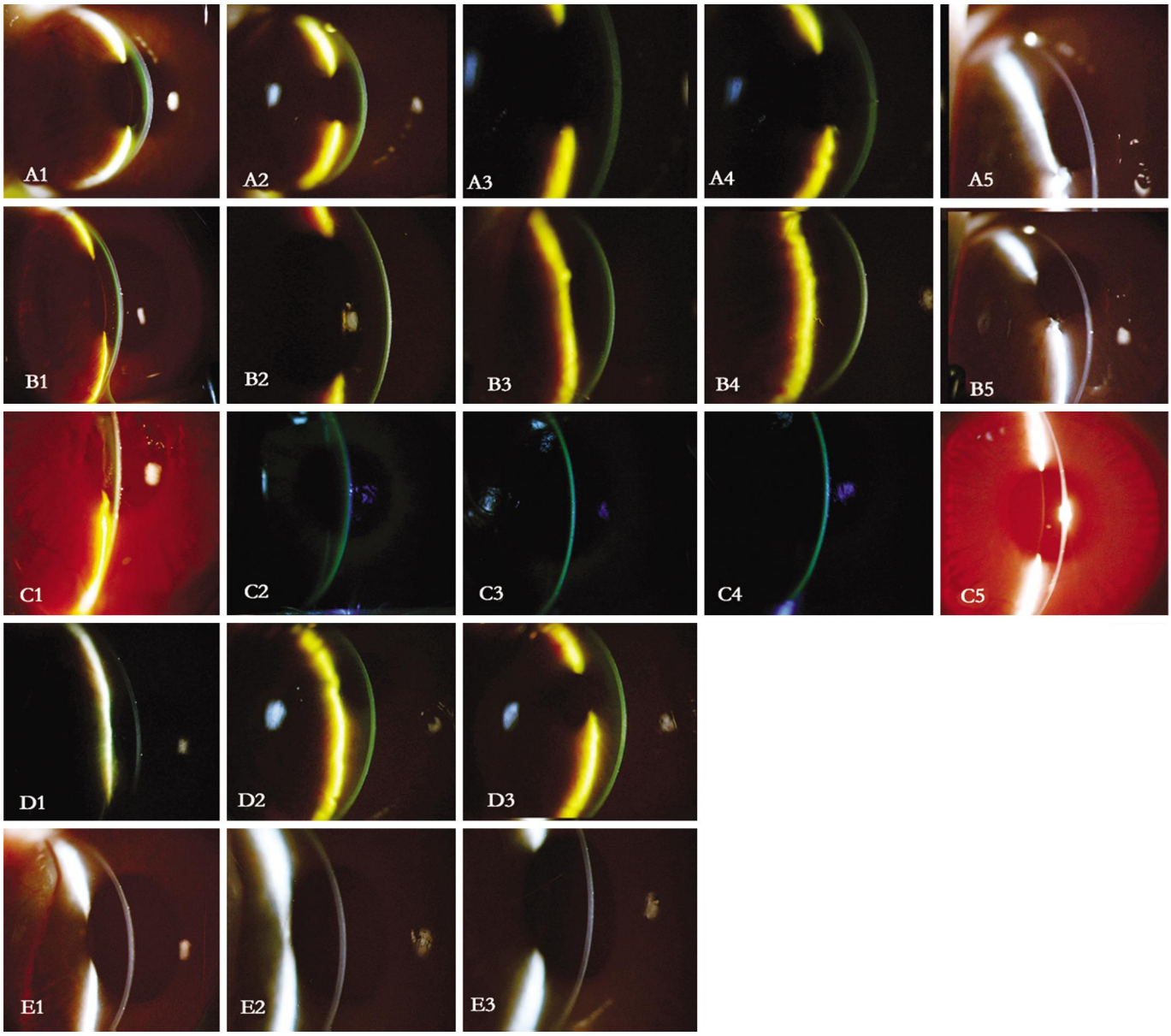

Figure 1 Photograph of eye anterior segment with different treatments A: Intracameral 0.5% riboflavin-BSS solution. A_1: 1min; A_2: 15min; A_3: 30min; A_4: 60min; A_5: 24h. B: Intracameral 1% riboflavin-BSS solution. B_1: 1min; B_2: 15min; B_3: 30min; B_4: 60min; B_5: 24h. C: Intracameral 2% riboflavin-BSS solution. C_1: 1min; C_2: 15min; C_3: 30min; C_4: 60min; C_5: 24h. D: Instillation 0.1% riboflavin-BSS solution without epithelium. D_1: 1min; D_2: 15min; D_3: 30min. E: Instillation 0.1% riboflavin-BSS solution with epithelium. E_1: 1min; E_2: 15min; E_3: 30min.

RESULTS

Photograph of Eye Anterior Segment At 30min, the degree of yellow staining in the corneal stroma increased with the increase in concentration of intracameral riboflavin. The amount of staining in the 1% group was similar to the de-epithelialized group. The staining in the 0.5% group was inferior to de-epithelialized group but still superior to the transepithelium group. The 2% group had slightly more staining than the de-epithelialized group. The yellow stain seemed continued for at least 30min. The inflammatory effusion in the anterior chamber only appeared with the 2% group. At 24h, the yellow change in the cornea for the three different concentrations groups and the inflammatory effusion in the 2% group disappeared (Figure 1).

Yellow Stained Corneal Stromal Buttons and Cartridge The corneal stromal buttons with a higher concentration riboflavin solution after intracameral injection were more yellow (Figure 2). The color change of the C18-SPE cartridge infused with different riboflavin supernatants for the HPLC determination had a similar trend (Figure 3). The 1% group was similar to the de-epithelialized group.

Riboflavin Concentrations in Corneal Stroma An enriching effect of riboflavin that was similar to the classical de-epithelialized CXL could be achieved with intracameral 1% riboflavin-BSS solution 0.1 mL at 30min (Table 1, Figure 4). The specimens for HPLC examination all had the epithelium and endothelium debrided, and the result indicated the enrichment of riboflavin in the corneal stroma.

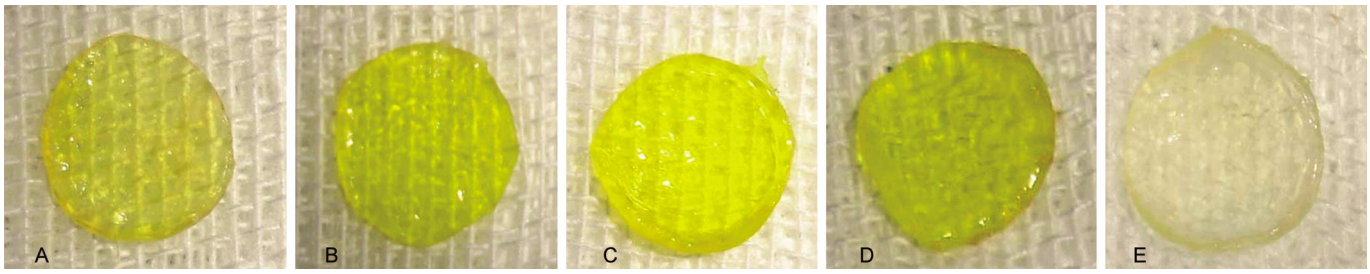

Figure 2 Yellow stained corneal stromal buttons at 30min A: Intracameral 0.5% riboflavin-BSS solution; B: Intracameral 1% riboflavin-BSS solution; C: Intracameral 2% riboflavin-BSS solution; D: Instillation of 0.1% riboflavin-BSS solution without epithelium; E: Instillation of 0.1% riboflavin-BSS solution with epithelium.

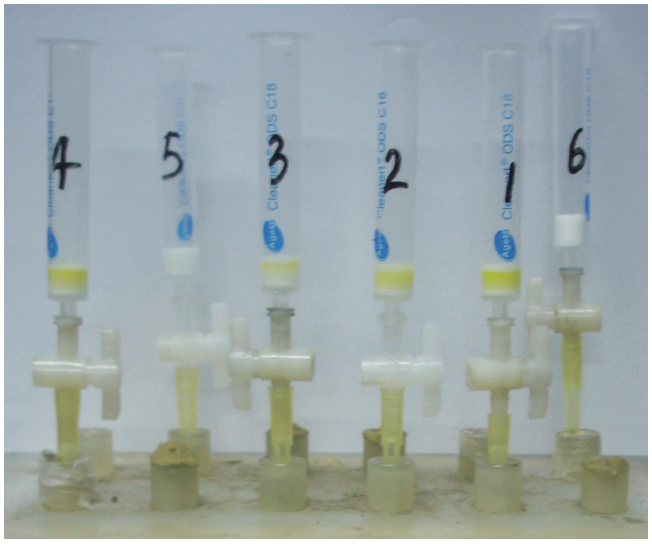

Figure 3 Yellow stained C18-SPE cartridge 1: Intracameral 2% riboflavin-BSS solution; 2: Intracameral 1% riboflavin-BSS solution; 3: Intracameral 0.5% riboflavin-BSS solution; 4: Instillation of 0.1% riboflavin-BSS solution without epithelium; 5: Instillation of 0.1% riboflavin-BSS solution with epithelium; 6: Untreated cartridge.

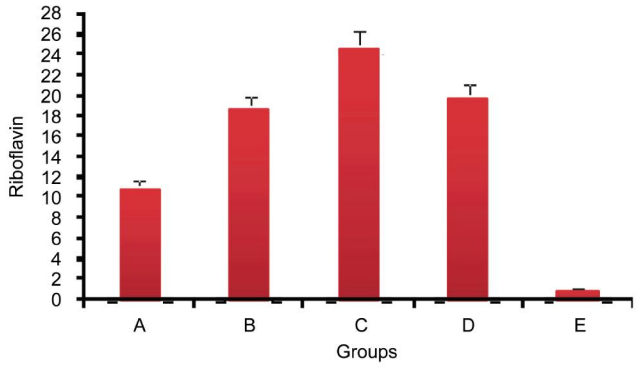

Figure 4 Riboflavin concentrations in the corneal stroma after different treatments at 30min A: Intracameral 0.5% riboflavin-BSS solution; B: Intracameral 1% riboflavin-BSS solution; C: Intracameral 2% riboflavin-BSS solution; D: Instillation of 0.1% riboflavin-BSS solution without epithelium; E: Instillation of 0.1% riboflavin-BSS solution with epithelium.

The method avoided the interference of riboflavin by the epithelium or endothelium.

Table 1 Riboflavin concentrations in the corneal stroma after different treatments at 30min by HPLC μg/g

Groups	$\bar{x} \pm s$	^1t	^1P
0.5%	11.19±2.19	7.599	0.000
1%	18.97±2.14	1.028	0.321
2%	25.08±3.45	3.236	0.006
De-epithelialized	20.18±2.53		
Transepithelium	1.13±0.05		

[1]Compared with de-epithelialized group.

DISCUSSION

The HPLC determination had a coincidental result with the degree of yellow staining of the corneal stroma. The results of this study showed that the penetrative effect of riboflavin through the corneal endothelium increased with the increase in concentration. Comparing the three types of riboflavin-BSS solution with different concentrations (0.5%, 1%, and 2%), the enrichments of riboflavin at 30min after intracameral injection all were superior to that with trans-epithelial instillation. The riboflavin concentrations in the 2% group and 1% group were obviously higher than 15 μg/g which was the theoretically safe and effective concentration for de-epithelialized CXL [7]. The duration of the enrichment lasted for at least 30min using this method; this was proven by the yellow staining of the corneal stroma at 60min. This ensured the stability of riboflavin during the UVA irradiation period. This penetration effect may possibly be related to the leaky corneal endothelial junctions [23]. The inflammatory effusion in the 2% group disappeared at 24h, which demonstrated the safety of the intracameral injection. Although the operation was risky and invasive [24], it might become an enhancing method that improves the corneal riboflavin concentration in transepithelial CXL.

As a sensitive method to determine the concentrations of riboflavin [25], the HPLC results may evaluate the level of riboflavin enrichment in the corneal stroma through different intensive methods of transepithelial CXL. In 2009, Baiocchi *et al* [7] reported the riboflavin concentrations in the corneal stroma with and without epithelium by HPLC. The sharp

difference (1:100) suggested the necessary of improving riboflavin penetration into the stroma by intensive methods of transepithelial CXL. At the same time, they concluded that the process needed debridement of the epithelium to avoid interference of riboflavin in the epithelium. We used a similar HPLC method with minor modifications for this determination. Meanwhile, the corneal buttons had the epithelium and endothelium debrided to ensure that the results reflected the riboflavin concentration of the corneal stroma because of the possibility of high concentrations in the endothelium after intracameral injection.

In this study, comparing with the HPLC results at 30min of Baiocchi *et al*[7] (95.60 ng/g and 24.06 μg/g for with and without epithelium), the riboflavin concentrations in the transepithelial and the de-epithelialized groups were higher and slightly lower, respectively. The first possible reason was the different riboflavin solutions which had different components, osmotic pressures, and mucosity levels; the riboflavin-BSS solution was used in this study but not the riboflavin-20% dextran solution because the latter solution might increase the ocular pressure. Another reason was that this experimental subject was the rabbit eye *in vivo* and not the corneas from donors or the eye bank *ex vivo*. The construction, the epithelial barrier function, and the dynamic state of the rabbit cornea *in vivo* impacted the results.

The major limitation of this study was the safety issue of the corneal endothelial cells. We believed that with the UVA exposure after this type of drug administration, the epithelium and stroma containg the riboflavin would absorb the UVA energy; therefore, the UVA levels that reached the endothelium will be lower. In this study, we observed that the anterior segment of the rabbit eyes in each group was normal after 24h. But endothelial cell examinations such as corneal confocal scanning imaging which may show subtle variations, the corneal thickness, and the intraocular pressure should be required for safety concerns. Even if we also include a UVA exposure test, this result will ensure the safety of this simple method.

There are still some questions from this study that need to be answered such as the pharmacokinetics of riboflavin and the influence on corneal periphery tissues. Simultaneously, if the plateaus of the absorption coefficient occurred when increasing the concentrations of riboflavin-BSS solution similar to instillation in the de-epithelialized group [26,27], we will need larger number of animal test to undertake HPLC determinations. Furthermore, the risk of intracameral injection suggested that the indications for clinical application must be strict. However, the significant enrichment of riboflavin in the corneal stromal after intracameral injection offered promising avenues for further research. Next, we would perform a relative test.

In conclusion, riboflavin can effectively penetrate into the corneal stroma through the endothelium after intracameral injection *in vivo*; it can even achieve the enrichment effect similar to that after the instillation on a de-epithelialized corneal surface.

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Conflicts of Interest: Li N, None; **Peng XJ,** None; **Fan ZJ,** None; **Pang X,** None; **Xia Y,** None; **Wu TF,** None.

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