The comparative safety of genipin versus UVA-riboflavin crosslinking of rabbit corneas

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Purpose: To investigate, after 24 h, the safety of genipin or ultraviolet A (UVA)-riboflavin crosslinking of keratocytes and endothelial cells.

Methods: Fifteen New Zealand white rabbits were selected and divided into a PBS group (five rabbits), a 0.2% genipin crosslinking (GP-CXL) group (five rabbits), and a UVA-riboflavin crosslinking (UVA-CXL) group (five rabbits). In the GP-CXL and PBS groups, 0.2% genipin or PBS was applied to the corneal surface of the right eyes. In the UVA-CXL group, a clinical crosslinking procedure was used. Before and after surgery, the operated eyes of each group were characterized with confocal microscopy, and the corneal buttons were excised for endothelium staining and electron microscopy.

Results: The corneal endothelial cell density of the GP-CXL, UVA-CLX, and PBS groups changed. There was a statistically significant difference in thickness and changes in corneal endothelial cell density between the UVA-CXL group and the PBS group (p<0.05), and between the UVA-CXL group and the GP-CXL group (p<0.05), but no statistically significant difference between the GP-CXL group and the PBS group. Confocal microscopy, transmission electron microscopy, and hematoxylin and eosin staining showed that there was keratocyte apoptosis in the anterior and middle stroma and endothelial cell damage in the UVA-CXL group. In the GP-CXL group, only active keratocytes were found and minimal endothelial cell damage.

Conclusions: Treatment of rabbit corneas with 0.2% genipin showed minimal toxicity toward keratocytes and endothelial cells. Genipin is safer than UVA-CXL for crosslinking of thin corneas.

Keratoconus typically is characterized by progressive corneal protrusion and thinning, leading to irregular astigmatism and impairment of visual function [1]. Management of keratoconus includes eye glasses, soft and hard contact lenses, and intracorneal ring segments. However, these methods only improve visual acuity, but do not stop disease progression [2] and have advantages and disadvantages. The most common treatment for advanced keratoconus has been either penetrating or deep anterior lamellar keratoplasty. Recent studies have reported that after ultraviolet A (UVA)-riboflavin corneal collagen crosslinking (often referred to as "UVA-CXL") of the corneal stroma, new chemical bonds can be formed between collagen fibril molecules, resulting in enhanced mechanical strength of the cornea [3-6]. UVA-CXL has been used clinically to treat keratoconus, and studies have reported that UVA-CXL can partially flatten or stabilize the corneal surface and stop keratoconus progression [6-8]. This procedure is thought to be the only one capable of altering the natural history of keratoconus [9].

Although UVA-CXL shows positive results, this treatment has limitations. First, it has been recommended that patients with thin (<400 µm) central corneas not undergo this procedure because the depth of UVA penetration may expose the endothelial cells to toxic photochemical damage. Kymionis et al. reported that UVA-CXL treatment of corneas with central pachymetry <400 µm results in a significant endothelial cell loss [10]. Second, because free radicals of oxygen occur with riboflavin photolysis, this crosslinking method has a negative effect on keratocytes, and it has been reported that keratocyte apoptosis occurs during treatment [11]. Furthermore, confocal microscopy of the cornea has revealed the absence of keratocytes at the anterior 300 µm of the stroma at 3 months after UVA-CXL treatment [12,13]. Third, UVA-CXL is not suitable for patients who have photosensitivity disorders. Finally, if the maximum keratometry (K) is more than 58 diopters, the patient's age is >35 years, or the corrected distance visual acuity is better than 20/25, the rate of UVA-CXL complications increases significantly [14].

Considering the safety concerns with UVA-CXL, previous studies have tried to identify safer and more effective crosslinking agents. Sung et al. reported that genipin is a natural crosslinking agent [15], with a cytotoxicity 5,000- to 10,000-fold less than glutaraldehyde [16]. Avila et al. used

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genipin to crosslink porcine corneas in vitro and reported that corneal collagen crosslinking with genipin produced a significant increase in biomechanical strength. After crosslinking, the stress of corneal stroma had a 218% increase compared with the control group with 0.25% genipin [17]. Further studies characterizing the efficacy and safety of 0.25% genipin and UVA-CXL for porcine corneal crosslinking in vitro showed that UVA-CXL and genipin treatments have similar cytotoxic effects on endothelial cells [18]. Recently, Avila et al. reported that 0.25% genipin can flatten the cornea with mean 4.4 diopters +/− 0.5 [SD] [19]. Avila et al. thought genipin could be useful in the treatment of corneal ectasia and in the modification of corneal curvature. In our group, Xiao-Min et al. applied 0.2% genipin on the rabbit cornea in vivo and found that genipin has less toxicity toward stromal cells and endothelial cells. Currently, few studies have reported the safety of genipin crosslinking of the thin cornea, and most of these studies have been conducted in vitro, with fewer in vivo studies. Therefore, in the following study, we applied 0.2% genipin on rabbit corneas in vivo and compared the toxicity on the corneal stroma and corneal endothelium with UVA-CXL treatment.

METHODS

Animals: All animal experiments were performed in accordance with the Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals (Vgkfcz-2006–398) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Peking University First Hospital Ethics Committee. Fifteen healthy female New Zealand white rabbits (3.0–3.5 kg) were used in the study. All animals were provided by the Peking University First Hospital Animal Center. The animals were subdivided into three groups consisting of a PBS group, a genipin crosslinking group (GP-CXL), and a UVA-CXL group, with five rabbits in each group.

Genipin crosslinking: Genipin (Wako Pure Chemical Industry, Osaka, Japan) was dissolved in an isotonic medium (PBS, 1X, pH 7.3±0.2, ZSGB-BIO, Beijing China) to a final concentration of 0.2%. Rabbits were anesthetized with an intravenous injection of 5% pentobarbital (provided by the Peking University First Hospital Animal Center), and then 8 mm of the corneal epithelium was removed by scraping the corneal central surface. The genipin solution was applied as a droplet every 2 min for 30 min to the operated eye. After surgery, PBS was used to wash the corneal surface and conjunctival sac, followed by application of a ofloxacin gel (Sinqi Pharmaceutical, Shenyang, China) to the operated eye to protect the cornea from infection.

UVA-riboflavin crosslinking: Rabbits were anesthetized with an intravenous injection of 5% pentobarbital. Eight millimeters of the corneal epithelium were removed by scraping the corneal surface, and then 0.1% riboflavin (Sigma-Aldrich, Darmstadt, Germany) dissolved in 20% dextran (Adamas, Shanghai, China) was applied to the cornea as a droplet every 5 min for 30 min, followed by 30 min of UVA exposure (365±5 nm, 3 mW/cm^2) using a light-emitting diode (Lamplic Technology, Shenzhen, China). After surgery, PBS was used to wash the corneal surface and the conjunctical sac. Ofloxacin gel was then applied to the operated eye to protect the cornea from infection.

PBS control group: Rabbits were anesthetized in the same manner as in the other groups, and the corneal epithelium was removed. PBS was applied as a droplet every 2 min to the operated eye for 30 min, followed by the same treatments as the other groups.

In vivo measurement of the corneal thickness and scanning confocal microscopy of rabbit corneas were performed before and after surgery. The corneal thickness of the operated eye was measured with ultrasound pachymetry (PachPen; Accutome, Malvern, PA), and confocal microscopy (Heidelberg Retina Tomograph II/Rostock Corneal Module; Heidelberg Engineering, Heidelberg, Germany) images were obtained from the epithelium and endothelium. Before the examination, each rabbit was anesthetized as previously described.

Endothelium evaluation: Animals were euthanized with an intravenous overdose injection of 5% pentobarbital 1 mg/kg. The corneal buttons were excised for endothelial staining and electron microscopy 24 h after surgery. The corneas were collected and divided equally into two parts. One half of the cornea was examined with alizarin red (Sigma-Aldrich) and trypan blue (Sigma-Aldrich) staining. First, the 2.5% trypan blue solution was dropped on the endothelium for 30 s, and then PBS was used to wash the trypan blue solution. Then 1% alizarin red was dropped on the endothelium for 2 min, and PBS was used to wash the alizarin red. After the observation, the tissue was used for histopathology staining. The other half was used for scanning and transmission electron microscopy.

Electron microscopy: Each group comprised five corneal buttons. Two buttons were used for scanning electron microscopy (SEM, Hitachi, H-450), and the other three buttons were used for transmission electron microscopy (TEM, JEOL-100CXII). The main procedure of the electron microscopy was performed by professional staff at our hospital.

In the SEM procedure, the corneal buttons fixed in 4% glutaraldehyde for more than 1 h, washed in a buffered

solution of 0.2% sucrose-cacodyl for 4−10 h, postfixed in 1% osmium tetroxide in veronal acetate buffer for 1 h, and dehydrated through an ethanol series. The samples were then dried and mounted on SEM stubs using carbon adhesive tabs. They were then sputter-coated with a 10-nm thick layer of gold and examined with a scanning electron microscope (Hitachi, H-450).

In the TEM procedure, the corneas were fixed in the 2% glutaraldehyde for more than 24 h at 4 °C, washed in PBS three times, postfixed in 1% osmium tetroxide for 2 h, dehydrated through an acetone series, and then embedded in Epon. Ultrathin Epon sections 50–70 nm thick were cut. The samples were contrasted with 5% uranyl acetate and lead citrate and examined with TEM (JEOL-100CXII).

Histopathological assay: The corneal buttons were fixed in 4% paraformaldehyde (Sinopharm Medicine Holding Company, Ningbo, China). Serially graded ethanol baths followed by xylene were used to dehydrate the tissues before they were immersed in paraffin wax. A hematoxylin and eosin stain was used before the examination and evaluation with microscopy.

Statistical analysis: The experimental group and the control group were compared with Bonferroni ANOVA. Statistically significant comparisons were identified using SPSS for Windows statistical software (ver. 20.0; SPSS Inc., Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

Changes in the corneal thickness of operated eyes: After surgery, there was eyelid swelling, conjunctival congestion, and corneal edema in all eyes of the GP-CXL, UVA-CXL, and PBS groups (Figure 1). Before surgery, there was no statistically significant difference in corneal thickness among the three groups. After surgery, the corneal thicknesses of all three groups increased dramatically, especially in the UVA-CXL group. In the UVA-CXL group, two of the rabbit corneas could not be measured with ultrasound pachymetry (measuring range, 300–1,000 µm). Confocal microscopy was used to determine the depth of the endothelial cells at a corneal thickness of 942–963 µm (Table 1). There was a statistically significant difference ($p<0.001$) in the thickness change between the UVA-CXL group and the GP-CXL group, and between the UVA-CXL group and the PBS group ($p<0.001$). There was no statistically significant difference between the GP-CXL and PBS groups.

Confocal microscopy: Figure 2A,C shows confocal micrographs of active keratocytes in the corneas of the PBS group and the GP-CXL group. The endothelial cells could not be clearly seen because of corneal swelling, but normal cellular morphology and borders were evident (Figure 2B,D). In the UVA-CXL group, no normal keratocytes were seen in the anterior and middle stroma (Figure 2E). Active keratocytes could be seen in the deep cornea near the endothelium, but normal endothelial cells and cell borders were not evident. Confocal microscopy showed that the endothelial cytoplasm was concentrated and showed high reflectance (Figure 2F).
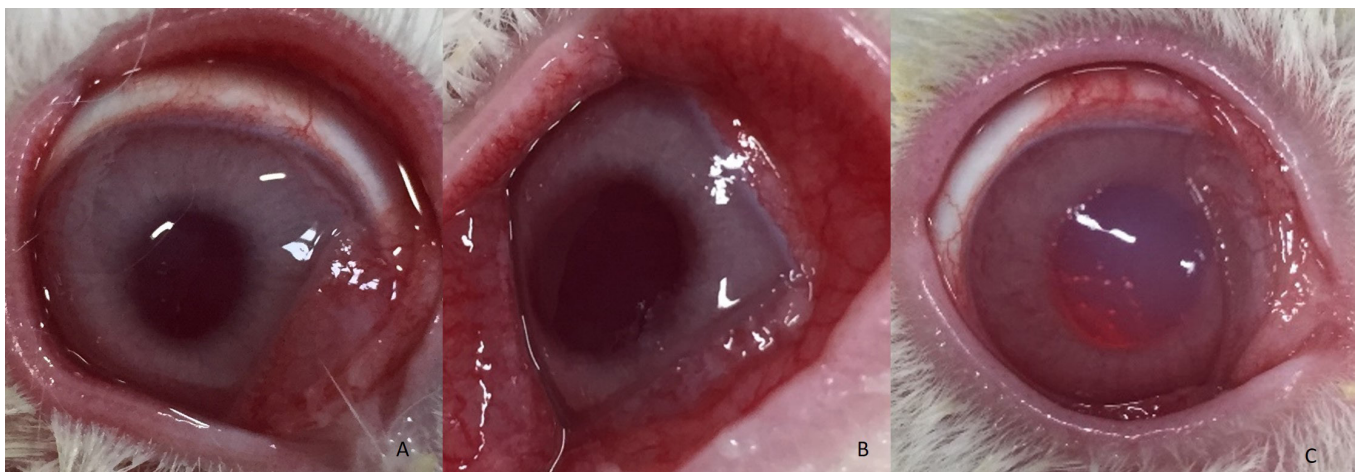

Figure 1. Colour photograph of rabbits'eye. Twenty-four hours after surgery, eyes of the PBS group **A**, the 0.2% genipin crosslinking (GP-CXL) group **B**, and the ultraviolet A (UVA)-riboflavin crosslinking (UVA-CXL) group **C** showed eyelid swelling, conjunctival congestion, and corneal edema.

TABLE 1. CORNEAL THICKNESS IN THE **PBS**, **GP-CXL**, AND **UVA-CXL** GROUPS BEFORE AND AFTER SURGERY.

Group	Before surgery (μm)	After surgery (μm)	Corneal thickness change (μm)
PBS (n=5)	378.4 ± 31.09	637.2 ± 77.61*	258.8 ± 92.06*
GP-CXL (n=5)	359.20 ± 13.29	645.6±57.33*	286.4±45.09*
UVA-CXL (n=5)	366.3 ± 9.18	852.6±98.52	486.4±93.42

PBS, phosphate-buffered saline; GP-CXL, 0.2% genipin crosslinking; UVA-CXL, UVA-riboflavin cross-linking. *$p<0.001$ compared with UVA-CXL goup.

Endothelial cell density was assessed using the software provided by the Heidelberg Retina Tomograph/Rostock Corneal Module. The corneal endothelial cell densities are listed in Table 2. Before surgery, no statistically significant difference was found among the three groups. After surgery, the endothelial cell density slightly decreased, with a statistically significant difference between the UVA-CXL group and the PBS group, and between the UVA-CXL group and the GP-CXL group ($p<0.05$).

Alizarin red and trypan blue staining: After trypan blue and alizarin red staining, normal hexagonal endothelial cells were present in the GP-CXL group (Figure 3B). Their borders were clear and maintained a hexagonal structure. There was no difference between the GP-CXL group and the PBS group
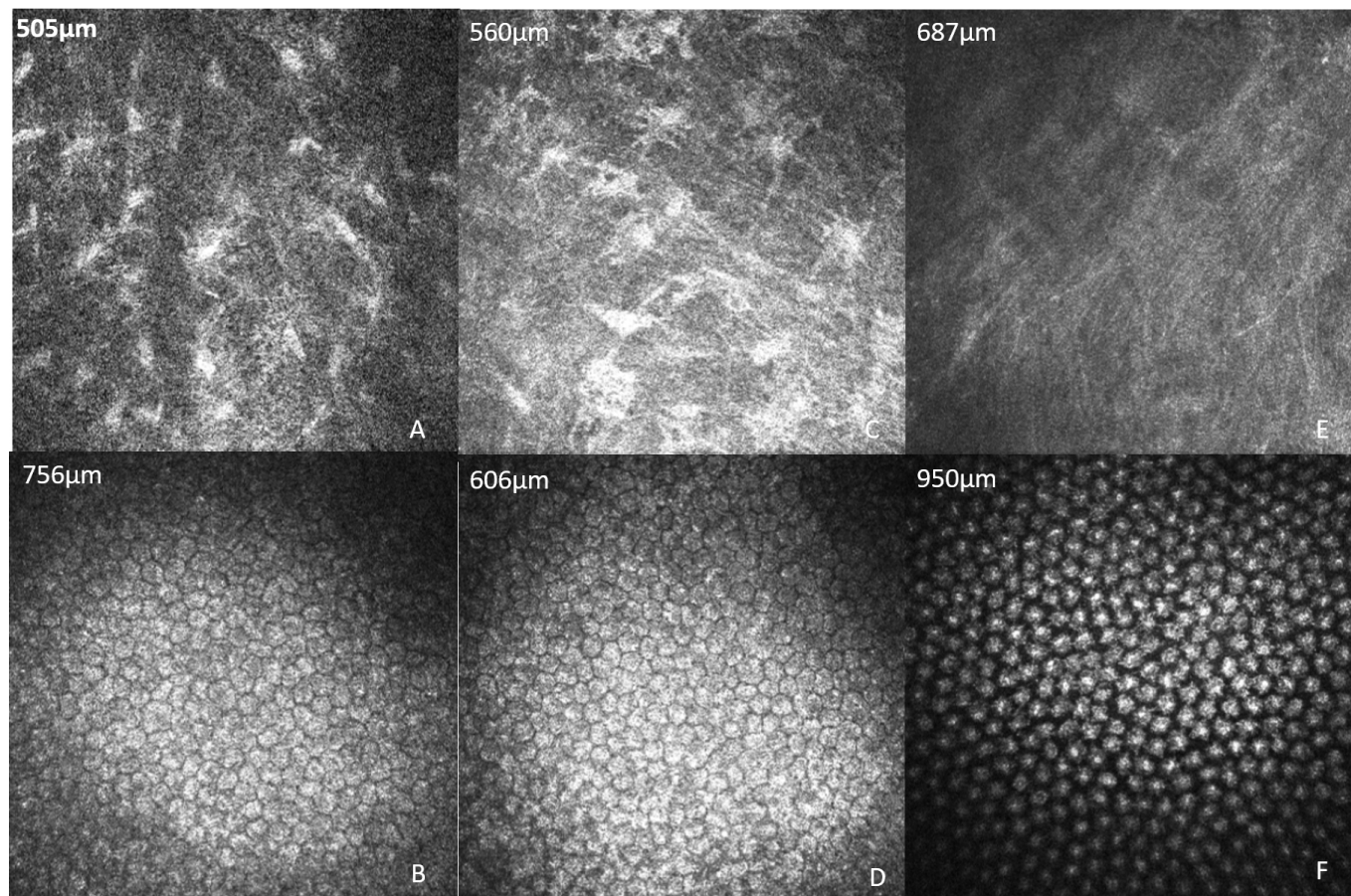

Figure 2. In vivo confocal microscopy of the corneal stroma and endothelium 24 h after surgery. **A**: In the PBS group and **C** the 0.2% GP-CXL group, active keratocytes can be seen. **B**: In the PBS group, normal endothelial cellular morphology and borders can be seen. **D**: In the GP-CXL group, normal cellular morphology and borders can be seen. **E**: In the UVA-CXL group, keratocytes are absent in the anterior and middle stroma. **F**: The endothelial cytoplasm is concentrated and shows high reflectance.
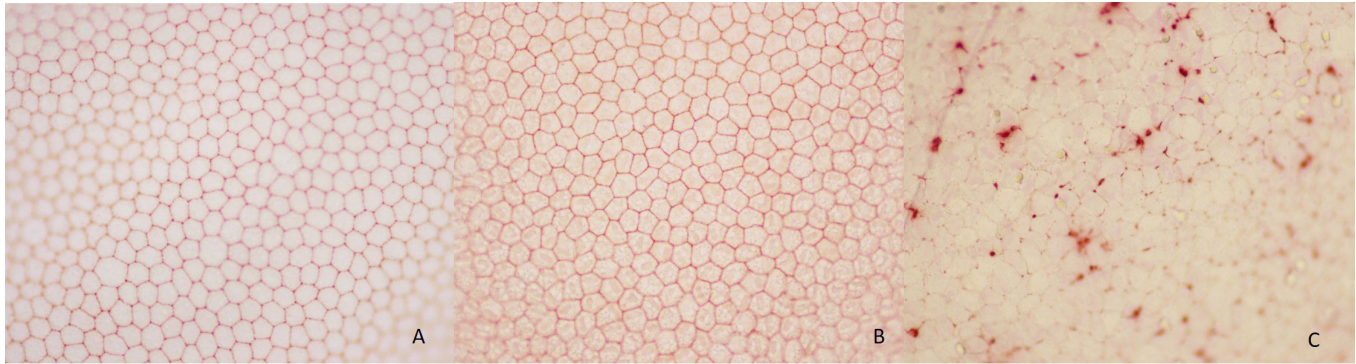
Figure 3. Alizarin red and trypan blue staining of the endothelium after surgery. **A**: The cells are seen as complete endothelial cells in the PBS group. **B**: The endothelial cell borders in the 0.2% GP-CXL group are clear. **C**: In the UVA-CXL group, many apoptotic cells are present.

(Figure 3A). In the UVA-CXL group, many apoptotic cells were present (Figure 3C).

Scanning electron microscopy: The SEM images in the PBS group showed that the endothelial cells maintained a hexagonal structure, the borders were clearly delineated and complete (Figure 4A), and the microvilli of endothelial cells were clearly maintained. In the GP-CXL group, solitary cell damage was occasionally seen, and the remaining endothelial cells had a hexagonal structure. The borders were clear and complete, and the cells were closely packed (Figure 4B). In the UVA-CXL group, substantial areas of endothelial cell damage were evident. The damaged endothelium contained many small cavities resembling holes in a sponge, and the normal hexagonal structure was absent. The cells around the damaged areas showed swollen changes and loss of microvilli (Figure 4C).

Transmission electron microscopy: TEM showed that the endothelium was arranged in a flat manner in the PBS group (Figure 5A) and in the GP-CXL group (Figure 5B). The nucleolus was normal, and its chromatin showed a normal distribution. In the UVA-CXL group, the endothelial cells were slightly bulged (Figure 5C–E), and swollen mitochondria could be seen. Some broken endothelial cells lost their normal hexagonal structures (Figure 5F).

The keratocyte structures in the PBS group and in the GP-CXL group were normal (Figure 6A,B). In the UVA-CXL group, keratocytes were absent in the anterior and middle stroma. Only some residual traces and apoptotic bodies were seen (Figure 6C). In the posterior stroma near the endothelium, apoptotic keratocytes showed the typical features of apoptotic chromatin condensation (Figure 6D).

Histological evaluation: The corneal stroma was edematous in all three groups. The stroma in the GP-CXL group (Figure 7B) was denser than in the PBS group (Figure 7A), and keratocytes were present throughout the stroma. In the UVA-CXL group, keratocytes were absent in the anterior and middle cornea stroma (Figure 7C).

DISCUSSION

There are currently numerous types of chemical crosslinking agents [20]. Genipin is one of these agents that is extracted from the fruit of *Gardenia jasminoides*. Genipin has been widely used as an antiphlogistic and cholagogue in herbal medicine [21]. Sung et al. reported that genipin is less cytotoxic than glutaraldehyde, and the in vivo biocompatibility of genipin is significantly better than that of glutaraldehyde [22,23]. Genipin is currently used for xenograft scaffolds of heart valves [24] and for treating liver fibrosis [25]. In

TABLE 2. CHANGE IN THE ENDOTHELIAL CELL DENSITY OF THE **PBS, GP-CXL,**
AND **UVA-CXL** GROUPS BEFORE AND AFTER SURGERY.

Group	Before surgery (cells/mm²)	After surgery (cells/mm²)	Change (cells/mm²)
PBS (n=5)	2946.74 ± 80.65	3074.86 ± 195.15	128.12 ± 185.38†
GP-CXL (n=5)	2817.57 ± 195.37	2966.77 ± 281.70	149.20 ±124.93†
UVA-CXL (n=5)	2761.59 ± 99.49	2551.35 ± 163.47	-210.24 ± 242.48

PBS, phosphate-buffered saline; GP-CXL, 0.2% genipin crosslinking; UVA-CXL, UVA-riboflavin crosslinking. †$p<0.05$ compared with UVA-CXL group.
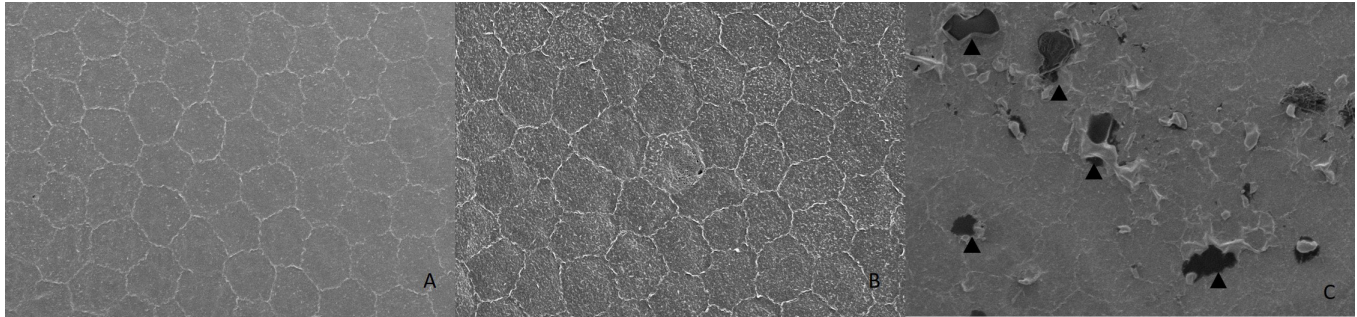
Figure 4. Representative SEM images of endothelial cells after surgery (1,000X). **A**: Endothelial cells in the PBS group appear the same as endothelial cells in the 0.2% GP-CXL group. **B**: In the GP-CXL group, solitary cell damage can be seen, with the remaining endothelial cells maintaining a hexagonal structure. **C**: In the UVA-CXL group, substantial areas of endothelial damage are seen. The damaged endothelium contains many small cavities (black triangle) resembling holes in a sponge, and the normal hexagonal structure is absent.

addition, Ai et al. reported genipin suppresses injury-induced conjunctival fibrogenic responses [26]. Grolik et al. reported that genipin-crosslinked chitosan blends can be used for corneal epithelial tissue engineering [27]. Liu et al. and Avila et al. reported that porcine corneas crosslinked by genipin had minimal keratocyte toxicity and had a similar stiffness as corneas treated with UVA-CLX [17,18,28].

This study used confocal microscopy, TEM, and histopathology to characterize the crosslinking of keratocytes. Keratocytes were absent in the anterior and middle corneal stroma in the UVA-CXL group. In contrast, only active keratocytes were present in the GP-CXL group. The corneal stroma comprises approximately 90% of the thickness of the cornea. The mean keratocyte density ranges from 20,000 cells/mm^2 to 30,000 cells/mm^2 [29-31], and keratocytes play an important role in the synthesis and secretion of stromal constituents. Furthermore, keratocytes in the stroma synthesize collagens and proteoglycans, and maintain long-range associations with assembled collagen fibrils through an extended network of cytoplasmic filopodia [32]. After UVA-CXL treatment, keratocyte apoptosis was found to a depth of 300 μm [11]. However, we still found that there was keratocyte apoptosis near the endothelium in the UVA-CXL group. Although the cell membrane was intact, there were numerous apoptotic bodies in the cell, and the nucleus exhibited karyopyknosis. We suspected that the depth of keratocyte injury was deeper than observed with histopathology and confocal microscopy. In the GP-CXL group, only active keratocytes were present,
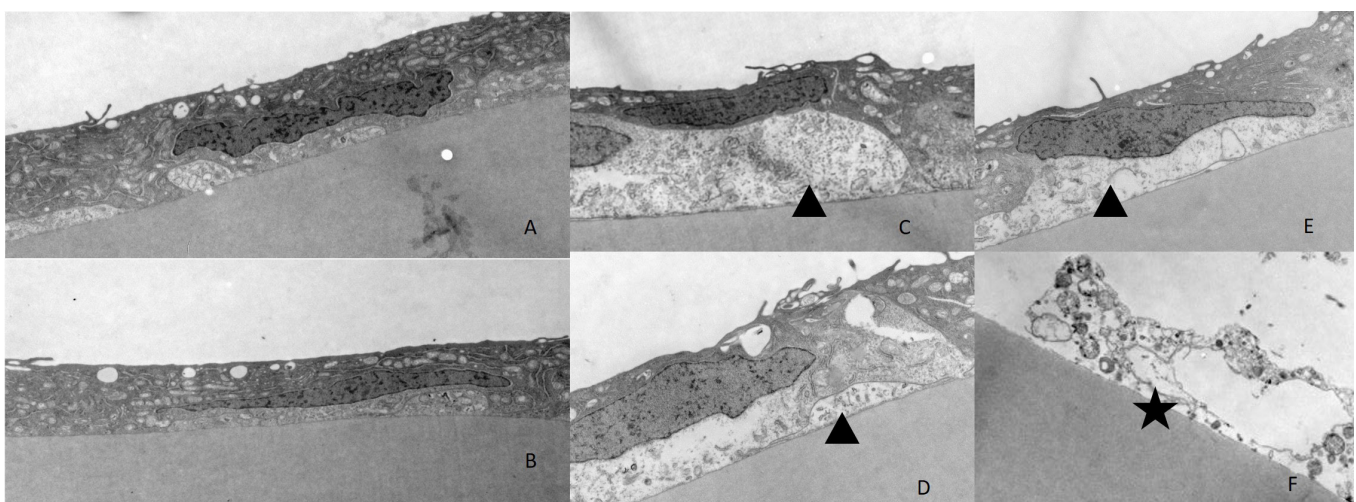

Figure 5. Representative images from TEM of endothelial cells after surgery (10,000X). **A**: In the PBS group, the endothelium is flatly arranged. **B**: In the 0.2% GP-CXL group, the endothelium is flatly arranged. The nucleolus chromatin shows a normal distribution and structure. **C–E**: In the UVA-CXL group, the mitochondria in the endothelial cells are swollen and the cristae are fractured (black triangle). **F**: Some broken endothelial cells have lost their normal hexagonal structures (black star).
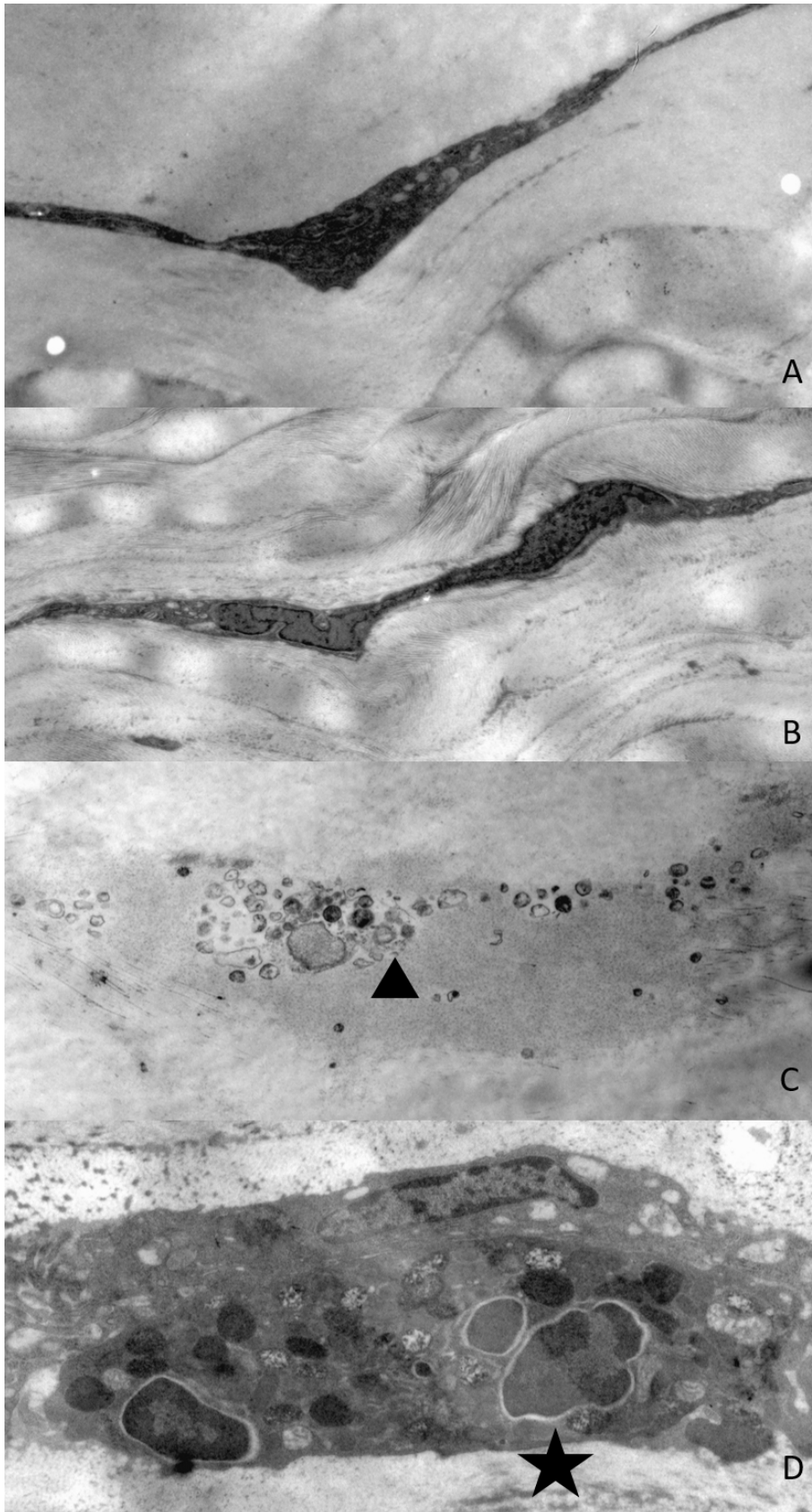
Figure 6. Representative images from TEM of keratocytes after surgery (10,000X). **A**: In the PBS group, the keratocyte structures are normal. **B**: In the 0.2% GP-CXL group, the keratocyte structures are normal. There is no difference between the GP-CXL and PBS groups. In the UVA-CXL group, the keratocytes are absent in the anterior and middle stroma. **C**: Only residual traces and apoptotic bodies (black triangle) are seen. D: In the posterior stroma, the apoptotic keratocytes show typical features of apoptotic chromatin condensation (black star).
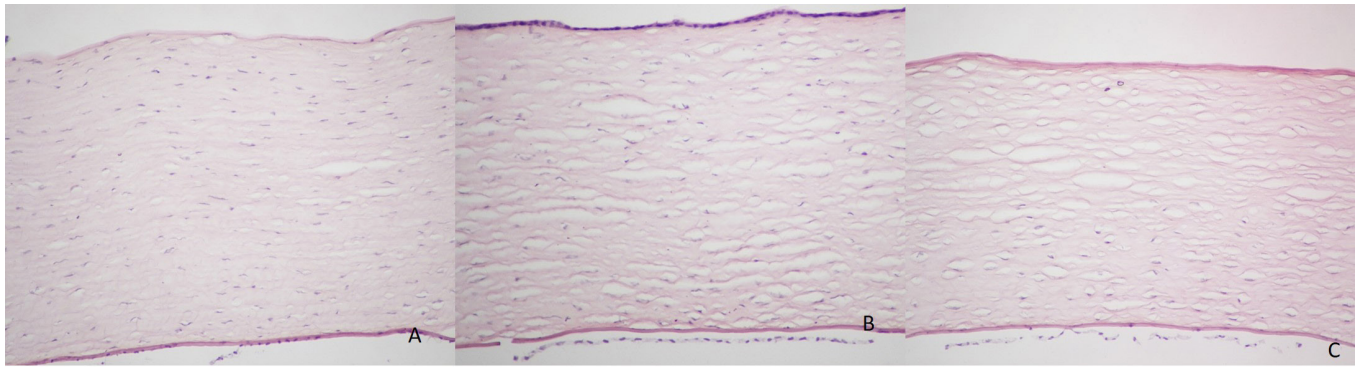
Figure 7. Representative images of the HE stain. In the PBS group (**A**) and the GP-CXL group (**B**), the corneal structures are normal (200X). **C**: In the UVA-CXL group, keratocytes are absent in the anterior and middle stroma.

suggesting that 0.2% GP-CXL is safer for stromal keratocytes than UVA-CXL.

In the present study, the mean corneal thickness of all groups was 367.93±20.45 μm. The corneal thickness of the experimental groups was 359.20±13.29 μm (GP-CXL) and 366.2±9.18 μm (UVA-CXL). In the GP-CXL group, almost no apoptotic endothelial cells could be detected. However, in the UVA-CXL group, much of the damaged endothelium could be seen. Previous studies suggested treatment of eyes with a central corneal thickness >400 μm, because of the known risks of endothelial damage [2]. Even in corneas greater than this thickness, there are reports of endothelial failure after treatment [33-35]. Some studies showed that during the process of UVA-riboflavin crosslinking, the use of energy may result in intraoperative thinning, exposing the endothelium to a high level of radiation despite an adequate thickness, especially if an eye speculum is used for a prolonged period during the procedure; this tends to promote stromal dehydration and thinning [36-41]. Nevertheless, there has recently been an increase in the use of UVA-CXL for eyes with thin corneas (<400 μm), using a variety of ingenious modifications of the originally described protocol. These protocols involve attempts to artificially or temporarily thicken the cornea before treatment. Consistent with this objective, sometimes the epithelium is not removed to provide additional thickness [28,42-46] or a hypotonic riboflavin solution is substituted for the usual isotonic solution to induce corneal swelling just before UV irradiation [47,48]. These procedures attempt to reduce toxicity to the endothelium. The major objection to leaving the epithelium intact is that it may substantially reduce the effectiveness of the procedure [49-51]. The success of the corneal swelling procedure is difficult to evaluate because of heterogeneity in protocols [34,52]. Furthermore, swelling will reduce the interfiber space and corneal density, which will lower the

potential efficacy of crosslinking [53-55]. Moreover, the majority of corneas for this potential treatment are barely thinner than the recommended minimum of 400 μm, with few corneas, including cases of severe thinning (<350 μm). In the present study, the cornea thickness of the GP-CXL group was approximately 350 μm (343 μm, 359 μm, and 350 μm). Based upon these findings, 0.2% genipin is a safe method for corneal crosslinking, even for thin corneas.

A limitation of this study is the lack of long-term results to confirm GP-CXL toxicity, to compare the long-term safety of the UVA-CXL and GP-CXL procedures. However, based upon the present results, we propose that GP-CXL for corneas with central pachymetry <400 μm is safe for keratocytes and endothelial cells. Therefore, genipin may provide an alternative approach for corneal crosslinking of thin corneas.

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