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A pilot study of genipin cross-linking effects on bullous keratopathy in rabbits

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京尼平交联治疗兔大泡性角膜病变的初步研究

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摘要

目的:评价京尼平交联治疗兔大泡性角膜病变的效果。

方法:将已建立大泡性角膜病变动物模型的9只雌性新西兰白兔随机分为3组:治疗组($n=3$),对照组($n=3$),空白对照组($n=3$)。其中治疗组:刮除角膜上皮后,应用0.25%的京尼平浸泡实验眼角膜40min(24℃);对照组:刮除角膜上皮后,应用生理盐水浸泡实验眼角膜40min(24℃);空白对照组:不进行任何处理。处理后2wk内进行以下检查:裂隙灯检查、中央角膜厚度(CCT)测量、体质量和应激反应评估、组织学检查和利用原位末端转移酶标记技术(TUNEL法)检测基质层细胞凋亡。

结果:与对照组和空白对照组相比,治疗组角膜水肿明显减轻、角膜上皮逐渐修复完整、角膜大泡消失、中央角膜角膜厚度显著下降($P<0.05$)、体质量显著增加($P<0.05$)。治疗组的实验动物活动性较处理前增加,反抗行为减少,攻击性减低。治疗组角膜基质层胶原纤维排列更为紧致、规则,可见蓝色条索状交联产物。治疗组角膜基质层均偶见凋亡细胞,对照组及空白对照组未明显见凋亡细胞。

结论:京尼平交联治疗可使兔大泡性角膜病变角膜水肿减轻,疼痛症状缓解,其可能的机制为京尼平交联使角膜基质层胶原纤维排列紧密,从而阻止角膜水肿和大泡的形成。

关键词:大泡性角膜病变;京尼平;胶原交联

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Abstract

• AIM: To evaluate the effects of genipin cross-linking on bullous keratopathy in rabbits.

• METHODS: Nine female New Zealand white rabbits with bullous keratopathy were used as an experimental model. They were randomized into three groups. Corneas in Group A (treatment group, $n=3$) were immersed in 0.25% genipin at 24℃ for 40min; those in Group B (control group, $n=3$) were immersed in 0.9% sodium chloride solution at 24℃ for 40min; and those in Group C (blank control group, $n=3$) received no treatment. Follow-up examinations were performed within 2wk after treatment, including slit-lamp microscopy, central corneal thickness (CCT), evaluations of body weight and stress responses, histopathological analyses, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) for detecting stromal cell apoptosis.

• RESULTS: Compared to Groups B and C, remission of corneal edema, corneal healing, disappearance of epithelial bullae, a significant decrease in CCT ($P<0.05$), and a significant increase in body weight ($P<0.05$) were found in Group A. Animals in Group A became more active and showed less aggression and violent resistance behavior. More regular and dense arrangement of collagen fibers in the corneal stroma and formation of blue strips of cross-linking products were observed in Group A. Cell apoptosis occasionally occurred in the corneal stroma of Group A, while no cell apoptosis was observed in Groups B and C.

• CONCLUSION: Genipin cross-linking treatment for bullous keratopathy in rabbits results in remission of corneal edema and relief of pain. We hypothesize that genipin cross-linking strengthens collagen fibers in corneal stroma to avoid the formation of corneal edema and bullae.

• KEYWORDS: bullous keratopathy; genipin; collagen cross-linking

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INTRODUCTION

Bullous keratopathy is a common condition resulting from endothelial decompensation[1], resulting in severe visual impairment and ocular irritations such as ocular pain, photophobia, and tearing[2]. Current treatment strategies for this disease consist of surgical procedures and conservative treatments[3-4]. The conservative treatments, including topical hypertonic saline eye drops and contact lens wear, partially reduce corneal edema and temporarily relieve ocular pain. However, the long-term effects of conservative treatments are unsatisfactory and may cause other complications. Penetrating keratoplasty[5-6] and cornea endothelial keratoplasty[7-9] are effective surgical procedures directed towards treatment of etiological factors, but their applications are limited by the graft source, surgical requirements, and economical factors[10]. In addition, patients with bad prospective vision after surgery and/or combined with wide-ranging adherent corneal leukoma and uncontrolled glaucoma are not suitable for these two surgical procedures. Other surgical options include conjunctival flaps, amniotic membrane transplantation, and anterior stromal puncture[1]. Recently, collagen cross-linking has been introduced as a new treatment for corneal disease[11-15]. Genipin is a cross-linking reagent commonly used in biomedical and material applications. It is reported to be as reliable as that of glutaraldehyde[14], and as a plant extract, genipin is much less toxic than glutaraldehyde[16]. Avila and Navia[17] treated porcine corneas *in vitro* with genipin to cross-link collagen, and found that the thickness of treated corneas decreased, biomechanical tensile strength increased, and resistance to bacterial collagenase increased. These results suggest that genipin can be used to treat keratectasia, corneal edema, cornea injuries, and other diseases, so we evaluated the effects of genipin cross-linking on bullous keratopathy in rabbits.

MATERIALS AND METHODS

Animals Nine female, 5 to 6-month-old New Zealand white rabbits weighing 2.2-3.0 kg (2.77±0.25 kg), and obtained and housed at the Peking University Experimental Animal Center were used as an animal model. Their left eyes had been treated to develop cornea bullous keratopathy 1wk before treatment as previously described[18]. A transparent corneal incision 1.5 mm wide was made at the 2:00 o'clock position near the corneoscleral limbus, and the endothelium was scraped away using a corneal endothelium clasp. All procedures were performed by a single surgeon (JB). Prior to these procedures, the animals were intravenously anesthetized with 5% sodium pentobarbital (0.6 mL/kg). Obvious corneal edema, gray corneal opacities, general cornea epithelial erosion, and bullae were observed in all of the treated (left) eyes. The research design was approved by the Ethical Committee of Peking University First Hospital. All animals in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Grouping and Treatment Protocols Experimental animals were randomly divided into three groups. In Group A (treatment group, $n=3$), after epithelial debridement, an 8 mm corneal trephine was placed on the cornea (the average horizontal and vertical diameters of rabbit corneas were 13.41 mm and 13.02 mm, respectively)[19], then filled with 0.25% genipin. Then the cornea was immersed in 0.25% genipin at room temperature (24℃) for 40min. The ocular surface was irrigated with 0.9% sodium chloride solution, and ofloxacin eye ointment was applied to the conjunctival sac after surgery. Chloromycetin (0.3%) eye drops were instilled three times per day and ofloxacin eye ointment was applied once every night to the operated eye from postoperative day 1 to prevent infection. In Group B (control group, $n=3$), the corneal trephine was filled with 0.9% sodium chloride solution, and the remaining treatment was the same as the treatment group. In Group C (blank control group, $n=3$), no treatment was performed.

Pre- and Postoperative Examinations Slit-lamp microscopy examinations were performed every day preoperatively and postoperatively to observe conjunctival and corneal inflammations in the anterior chamber of the experimental eyes. Central corneal thickness (CCT) was measured preoperatively and postoperatively by ultrasound biomicroscopy every 3d. Body weight and stress response were measured according to signs associated with pain or distress in rabbits as previously described[20]. Body weights of all experimental animals were measured preoperatively and postoperatively daily. Their activities, food consumption, behavioral changes, and vocal changes were also observed to indirectly evaluate their pain.

Histopathological Analyses One rabbit was randomly chosen from each group on postoperative day 7 and euthanized under anesthesia using an intravenous overdose injection of 5% sodium pentobarbital. All remaining rabbits were euthanized in the same manner on postoperative day 14. The cornea samples were quickly extracted and fixed in 4% paraformaldehyde for 24h at 4℃. The central 8 mm of the cornea was embedded in Tissue-Tek optimum cutting temperature compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen. Then all full-thickness corneas were vertically sliced into frozen sections 7 μm thick or 10 μm thick. The 7 μm sections were used for hematoxylin and eosin staining, while the 10 μm sections were used for the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect cell apoptosis.

The Nikon i50 Microphoto Collection System (Nikon, Tokyo, Japan) was used to collect all of the 3'-diaminobenzidine (DAB) staining photographs, containing brown areas representing areas of cell apoptosis.

Image-Pro Plus 6.0 medical image analysis software (Media Cybernetics, Rockville, MD, USA) was applied using half quantitative analyses for DAB staining sections. The apoptosis percentage of every section was obtained microscopically at 100× magnification in the following manner. More than two of the most stained visual fields were selected for analyses in

every section, and in every visual field an area 500 μm in diameter was used as the counting area. The apoptosis percentage of this counting area was calculated as the ratio of apoptotic cells in the stroma of the counting area to the total number of stromal cells in the counting area. The average apoptosis percentage of every sample was the mean value of the counting area values.

Statistical Analysis Statistical analyses were performed using SPSS, version 14.0 statistical software for Windows (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the least significant difference t-test were used for comparisons of CCTs and body weights in different groups. The rank sum test was used for comparative studies of the apoptotic index in different groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

Slit-lamp Microscopy At day 1 after cross-linking, mild bulbar conjunctival edema and hyperemia, a light blue cornea, and light blue hair that had been exposed to cross-linker on the eyelid margin were observed in Group A (Figure 1A). The light blue hair faded by day 3 after cross-linking (Figure 1B). The light blue cornea persisted throughout the entire observation period of 2wk. Significant recession of corneal edema, disappearance of epithelial bullae, and cornea epithelial healing were also observed in Group A (Figure 1). There were no obvious changes in the conjunctiva and cornea in Groups B and C, and epithelial bullae and corneal edema were continually found in these two groups (Figure 2). Inflammation in the anterior chamber of all of the experimental eyes was not observed by slit-lamp microscopy.

Central Corneal Thickness Table 1 shows the mean CCT values of each group at different preoperative and postoperative time points. There were no significant differences among these groups before treatment ($P>0.05$). Compared to CCT values in Groups B and C, significant decreases in CCT were observed at day 3, day 6, day 9, day 12, and day 14 in Group A (all, $P<0.05$). There were no significant differences between Group B and Group C ($P>0.05$).

Body Weight Table 2 shows the body weights of animals in each group at different preoperative and postoperative time points. There were no significant differences among these groups before treatment (all, $P>0.05$). Compared to the body weights of animals in Groups B, significant increases were observed in Group A animals from day 4 to day 14 ($P<0.05$). Compared to Group C, significant increases were observed in Group A from day 1 to day 14 ($P<0.05$). There were no significant differences in the body weights of animals in Group B and Group C ($P>0.05$).

Stress response rabbits in Group A spent more time moving in the cage and consuming food, and they ate more food, after cross-linking, compared to pre-crosslinking conditions. They also became less recalcitrant and showed an absence of growl or aggression when they were seized and medicated, with a behavior that resembled healthy New Zealand white rabbits. However, no obvious behavioral changes were observed in Groups B and C.

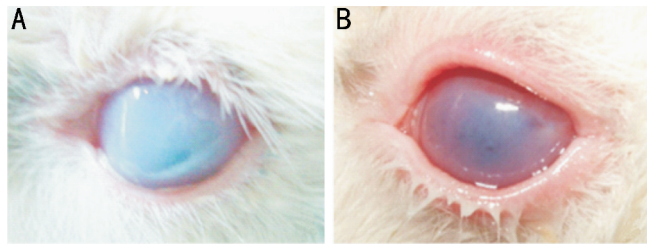

Figure 1 Group A eyes after cross-linking A: Cornea and hair of the eyelid margin were light blue at day 1 after cross-linking; B: Recession of corneal edema and disappearance of epithelial bullae were observed at day 3 after cross-linking. This appearance lasted for the entire observation period.

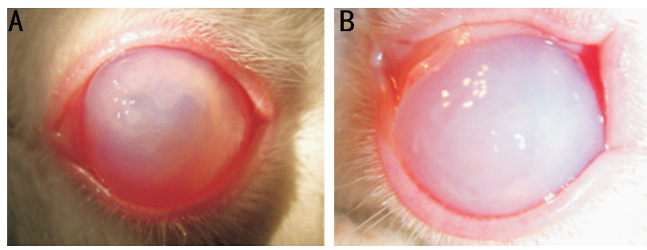

Figure 2 Cornea epithelial bullae and corneal edema persisted in Group B (A) and Group C (B) at day 14 after treatment.

Table 1 Central corneal thickness of each group at different preoperative and postoperative time points

Time	Central corneal thickness (μm)		
	Group A	Group B	Group C
Day 0	2356.7±434.7	2376.0±219.6	2439.3±225.4
Day 3	1361.0±506.5	2321.0±631.7	2542.3±259.7
Day 6	1243.7±328.9	2352.7±666.7	2437.3±252.9
Day 9	1336.0±345.1	2587.5±137.9	2454.5±147.8
Day 12	1293.0±298.9	2498.5±142.1	2454.0±154.1
Day 14	1281.0±284.0	2499.0±140.0	2462.0±151.3

Histology Normal rabbit corneas exhibited complete structures, with a regular arrangement of collagen fibers in the stroma and normal cells under light microscopy (Figure 3A), while the loss of the epithelium was observed in all three groups (Figure 3B-3D). In Group A, collagen fibers in the stroma were regularly arranged with little lymphocyte infiltration, and blue strips of cross-linking products (Figure 5, left, black arrows) were observed in the anterior two-thirds of the cornea (Figure 4). In Group B, a loose arrangement of collagen fibers in the stroma, many vacuoles, and little lymphocyte infiltration among the fibers were observed (Figure 5). The histological appearance of the blank control group was the same as the control group. There were no significant differences between the week 1 samples and week 2 samples within the same group.

Apoptosis of Stromal Cells The TUNEL assay was used to detect cell apoptosis. Corneal stromal cell apoptosis percentage in Group A, Group B and Group C was (1.0±0.1)%, (0.0±0.0)% and (0.0±0.0)%. There was a statistically significant difference in apoptosis percentage among these groups ($\chi^2=7.714$; $P=0.021$).

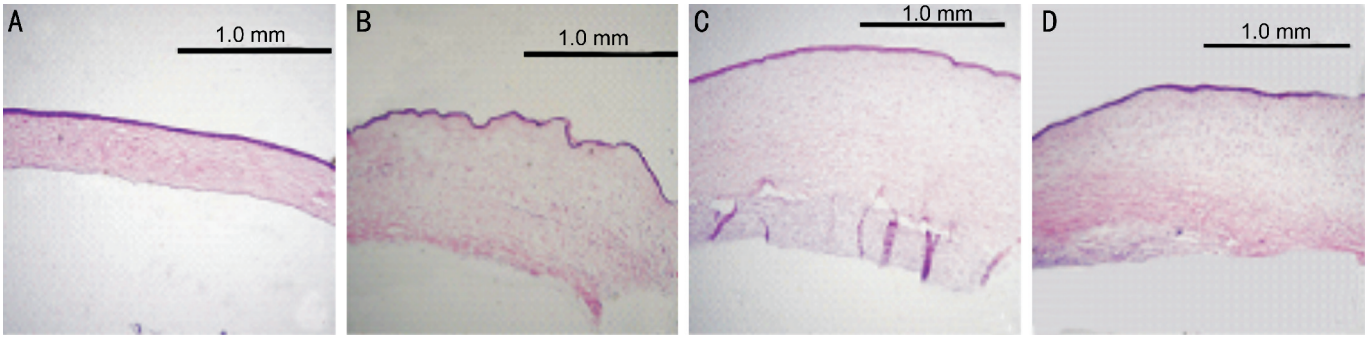

Figure 3　Histological examinations in different groups (week 2, hematoxylin and eosin staining, ×40)　A: normal cornea; B: Group A; C: Group B; D: Group C.

Table 2　Body weights of each group at different preoperative and postoperative time points

Time	Body weight (kg)		
	Group A	Group B	Group C
Day 0	2.60±0.34	2.77±0.21	2.93±0.58
Day 1	3.52±0.21	3.07±0.12	3.00±0.10
Day 2	3.57±0.25	3.07±0.12	3.00±0.10
Day 3	3.57±0.25	3.10±0.17	3.07±0.06
Day 4	3.67±0.15	3.13±0.23	3.10±0.00
Day 5	3.73±0.06	3.13±0.23	3.07±0.06
Day 6	3.70±0.10	3.17±0.21	3.07±0.06
Day 7	3.75±0.07	3.00±0.00	3.10±0.00
Day 8	3.75±0.07	3.00±0.00	3.05±0.21
Day 9	3.75±0.21	3.00±0.00	3.05±0.07
Day 10	3.75±0.21	2.90±0.14	3.00±0.00
Day 11	3.80±0.28	2.80±0.28	3.00±0.00
Day 12	3.80±0.28	2.85±0.21	2.90±0.14
Day 13	3.80±0.28	2.80±0.14	2.85±0.07
Day 14	3.80±0.28	2.75±0.21	2.80±0.00

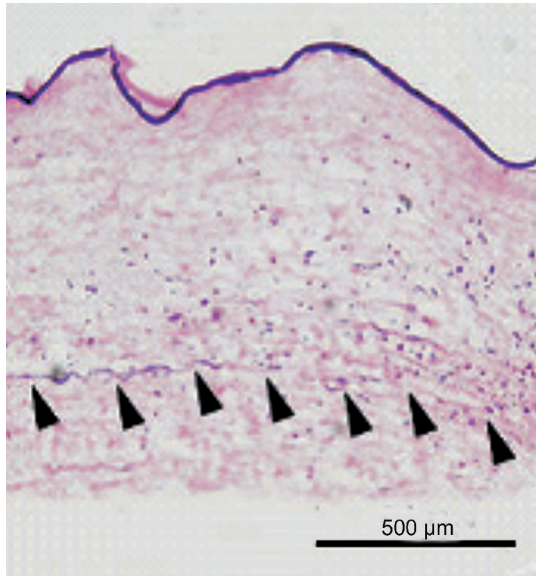

Figure 4　Histological slices ofthe cornea in Group A (week 2, hematoxylin and eosin staining, ×100)　Collagen fibers in the stroma were regularly arranged and little lymphocyte infiltration and blue strips of cross-linking products were observed in the anterior two-thirds of the cornea.

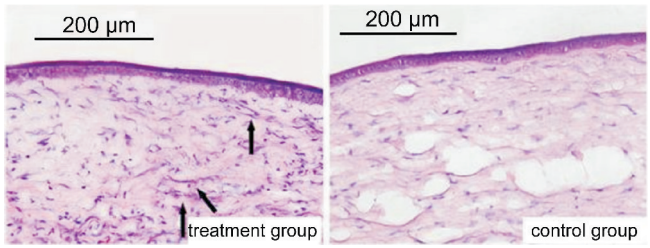

Figure 5　Histological slices inGroup A and Group B (hematoxylin and eosin staining, ×200)　Group A (left), collagen fibers in the stroma were regularly arranged and little lymphocyte infiltration and blue strips of cross-linking products (black arrow) were observed. Group B (right), there was a loose arrangement of collagen fibers in the stroma, and many vacuoles and little lymphocyte infiltration among the fibers were observed.

DISCUSSION

Genipin is extracted and purified from geniposide. As a common cross-linking reagent, it is widely used in biomedical and material applications[21]. In this study, 0.25% genipin, 24℃, and 40 min of cross-linking were considered the optimum cross-linking parameters. Genipin cross-linking resulted in a dense arrangement of collagen fibers in the corneal stroma, resulting in decreased corneal thickness, recession of corneal edema, improvement of corneal bullous changes, and relief of corneal epithelial erosion. Avila and Navia[17] characterized the effects of genipin cross-linking in porcine corneas *in vitro*, and Wollensak *et al*[22] reported the clinical effects of riboflavin/UVA cross-linking in bullous keratopathy cases. Our findings are consistent with the results of both of those studies. Genipin cross-linking was also effective in a living animal model, suggesting that it might be a reliable treatment for bullous keratopathy.

Except for the CCT, changes in the responses of experimental animals related to pain were also assessed after genipin cross-linking. Because it is difficult to directly evaluate the pain and stress in animals, we characterized their activity, food consumption, behavioral changes, and vocal changes to indirectly evaluate their pain responses. Body weights were sensitive and quantitative values that could be used for statistical analyses. Ocular pain is another symptom of bullous keratopathy, whose influence on quality of life may be greater than that of visual impairment resulting from corneal edema. Past animal studies of bullous keratopathy have not included an assessment of pain. We hope that a reasonable ocular pain

scale for animals can be designed in the future to directly evaluate pain and stress. In our study, the animals in the treatment group had increased body weight and showed decreased responses related to pain after genipin cross-linking, which indirectly confirmed that genipin cross-linking could relieve the ocular pain of bullous keratopathy.

Glutaraldehyde is a common cross-linking reagent that is 10 000-fold more toxic than genipin. Cells exposed to genipin retained a good proliferative ability, and new collagen fibers were produced in previous *in vitro* studies of genipin cross-linking toxicity[23]. In our study, apoptotic cells were occasionally observed in the corneal stroma after cross-linking in the treatment group, whose apoptosis percentage was (1.0±0.1)%. Genipin cross-linking is much safer than riboflavin ultraviolet-A cross-linking treatment that results in apoptosis and the disappearance of almost all cornea stromal cells[24].

Blue strips of cross-linking products were observed in corneal stroma after cross-linking in the treatment group, resulting in a light blue color for the cornea and the vicinal fur. Previous studies have reported that the blue substance resulting from the reaction between genipin and amino acids (mainly arginine, lysine, and oxylysine) or proteins is nontoxic, can be used as a food dye, and can be easily eluted by organic solvents[25-26]. In future studies, the observation period should be prolonged to determine whether the light blue cornea fades like the light blue fur. The blue substance was present in the anterior two-thirds of the corneal stroma after cross-linking. We assumed that the 0.25% genipin did not permeate throughout the cornea, so there was little possibility of cytotoxicity to the corneal endothelium, lens, and retina. However, another possible explanation was that the corneal thickness increased by 6- to 8-fold because of severe corneal edema in the experimental animal model, although this reaction is not common in the clinic. However, a series of studies on genipin toxicity related to corneal endothelium, lens, and retina should be conducted before genipin cross-linking is clinically used.

Genipin cross-linking treatment for bullous keratopathy in experimental animals resulted in recession of corneal edema and relief of pain, with no severe adverse effects on other ocular tissues. In the future, genipin cross-linking treatment could be an alternative to penetrating keratoplasty for treating bullous keratopathy with irreversible visual impairment such as severe traumatic bullous keratopathy, and bullous keratopathy in absolute glaucoma. As an initial study, there were many limitations such as small sample size and short observation periods after cross-linking. Therefore, further studies with larger sample sizes, different genipin concentrations, different cross-linking times, different cross-linking temperatures, and longer follow-up times are necessary.

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