

## Original Article

# Inhibitory effect of trichostatin on allograft rejection of corneal transplantation in rats

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**Abstract:** Background: Using a rat penetrating keratoplasty model, this study aims to explore the inhibitory effect of hachimycin on corneal graft rejection, to provide new basis for its clinical application. Materials and methods: Female adult Sprague-Dawley (SD) rats weighing between 220-250 g were used as acceptors and male or female Wistar rats weighing between 220-250 g were used as donors. The rats with a successful keratoplasty were randomly divided into 3 groups with 10 rats in each group. Group A: penetrating keratoplasty group; Group B: penetrating keratoplasty followed by the application of control eye drops containing eye drops matrix dissolved in 20 g/L DMSO and 900 mL/L artificial tear; Group C: penetrating keratoplasty followed by the application of 0.5 g/L hachimycin eye drops. Hachimycin was dissolved in vitamin E to obtain an eye solution with a pH value of 6~7, and stored at 4°C. The local application of hachimycin eye drops started 5 days after the keratoplasty surgery, 5 times per day until the onset of rejection response. At 4 days after the keratoplasty surgery, slit-lamp microscope was used to observe the transplanted cornea once every two days, and a rejection index (RI) of 0-12 was obtained according to the three graft components represented by corneal transparency, edema, and corneal neovascularization. Results: Penetrating keratoplasty was successfully performed on all the 3 groups of rats. Five days after the keratoplasty, both the transparency and the implant edema showed a score of 1-2 degrees in group A and B. Two weeks later, both these two grafts components increased to a score of 2-3 degrees in group A and B, with an active neovascularization. The group C also showed a transparency and implant edema of 1-2 degrees five days after the keratoplasty surgery. However, a transparent implant without edema was observed in group C two weeks after the keratoplasty surgery. In addition, the newly formed blood vessels disappeared and the retina appeared in a good status and in the correct position. The corneal transparency, edema, corneal neovascularization, and total RI scores of the 3 groups clearly indicated that the group B showed an improvement compared to the group A ( $P < 0.05$ ), since in group B the new vessels were only distributed in the corneal limbus at five days after the surgery. However, at two weeks after the surgery no statistically significant difference in neovascularization degree was observed in group B when compared with group A, while a statistically significant decrease of neovascularization was observed in group C ( $P < 0.05$ ). Conclusions: Hachimycin may inhibit the rejection responses after penetrating keratoplasty by the reduction of corneal edema, transparency and neovascularization.

**Keywords:** Trichostatin on allograft rejection, corneal transplantation, neovascularization, corneal edema, rejection index

## Introduction

Corneal transplantation is an effective way to treat various corneal diseases with the highest success rate among tissue and organ transplants. Immunological rejection of the graft remains the main reason for the corneal transplant failure. It has been reported that the incidence of rejections in corneal transplant is approximately 10%~30% [1]. In situations such as vascularization, serious infection, large graft

cornea transplant, and transplant surgery for the second time, the rejection rates are up to 60% [2]. To date, ciclosporin A (CsA) is the drug commonly used for suppressing the immune rejection after organ transplantation. However, if used as topical eye drops, it is difficult for CsA to penetrate the cornea to achieve an effective concentration for intraocular medication. In case of systemic administration, the side effects are significant and they cannot be ignored. Fungal keratitis possesses the highest

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incidence among infectious keratitis in China, representing the 60% of infectious keratitis [3]. The high risk factors include, but are not limited to, trauma, fungal keratitis due to operations such as photorefractive keratectomy and penetrating keratoplasty [4]. In the past, the lack of an effective anti-fungal eye medicine resulted in a poor prognosis and a high rate of blindness. In this study, rat was used as a penetrating keratoplasty model to study the inhibitory effect of topical application of Trichostatin on corneal allograft rejection, to provide new basis for its clinical applications.

### Materials and methods

#### *Experimental animals*

Adult male and female Sprague-Dawley (SD) rats, weight between 220 to 250 g, were used as recipients. Male and female Wistar rats, weight between 220 to 250 g, were used as donors. The SD and Wistar rats were provided by Xinjiang Hospital of Ophthalmology.

#### *Penetrating corneal transplant surgery*

Rats were anesthetized by an intraperitoneal injection of chloral hydrate 10% (35~42 mg/kg), pupil was dilated, and the penetrating corneal transplant surgery was conducted on the right eye of the recipient rat. The diameter of the graft was 3.5 mm and the diameter of the engraftment bed was 3.0 mm. Eight (8) to 12 interrupted sutures were applied using 10-0 nylon thread (Ethicon, United States). The suture nodes were exposed without coverage. After the operation, 2000 U Gentamycin was injected under the conjunctiva to avoid blepharitis. The rat model of corneal transplantation was then established. The operation was only conducted on the right eye of each rat to ensure that the rats can still see and feed properly.

#### *Experimental animal groups and reagents*

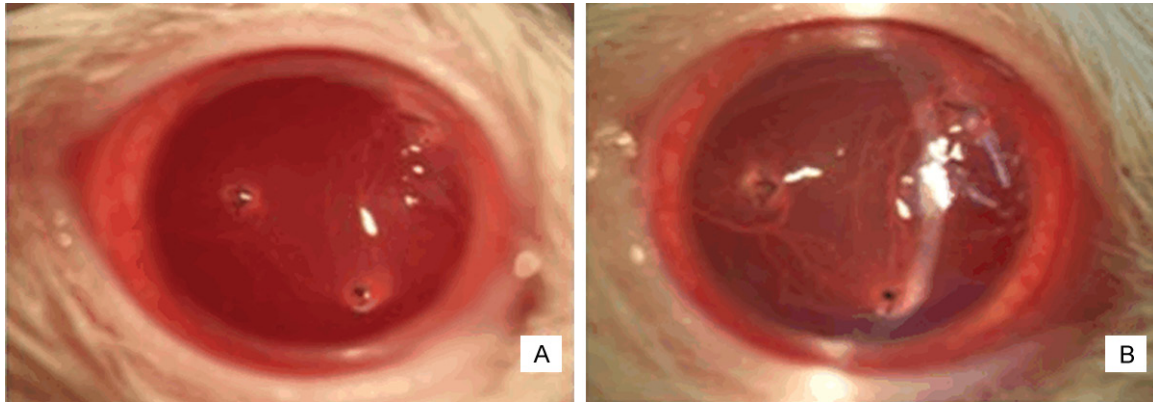
Five days after the corneal transplant, the animals with iridocyclitis, hyphema, and eye lens opacity were excluded. The animals with succeeded corneal transplant surgery were randomly divided into three groups, with 10 animals in each group. All groups were represented by allogenic corneal transplants with Wistar rats as the donor and SD rats as the recipient. Five days after the operation, different ophthal-

mic solutions were applied 5 times a day until the rejection of the corneal transplant occurs. Group A: penetrating keratoplasty. Group B: penetrating keratoplasty + placebo ophthalmic solution (containing only the matrix components, such as DMSO 20 g/L and artificial tear 900 mL/L). Group C: penetrating keratoplasty + 0.5 g/L trichomycin ophthalmic solution prepared by dissolving trichomycin in Vitamin E solution with a pH value of 6~7, and kept at 4°C as a stock. All the ophthalmic solutions were prepared in the pharmaceutical preparation lab in Xinjiang Hospital of Ophthalmology. Heat was used for solution sterilization and the pH value of all the ophthalmic solutions were set at 6~7. At the beginning of the 4<sup>th</sup> day post operation, slit-lamp microscope was used to observe the engrafted cornea once every two days. Using the degrees of transparency of the cornea, edema, and the formation of new blood vessels as the indicators, the graft rejection index (RI) (from 0 to 12) was recorded.

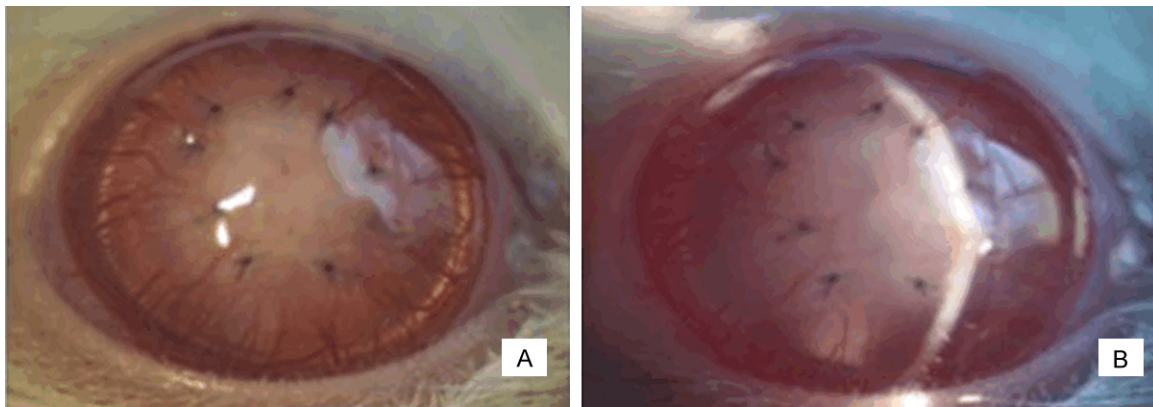
#### *Rejection observation and the judging criteria*

The scoring method of Holland *et al.* [5] was used as a reference to record various corneal parameters. (1) Transparency of the cornea: complete graft transparency was scored as 0 point; mild graft opacity was scored as 1 point; increased opacity with a still visible iris texture was scored as 2 points; increased opacity with a still visible pupil was scored as 3 points; full opacity and invisible anterior chamber was scored as 4 points. (2) Corneal edema: graft with no edema was scored as 0 point; matrix mild thickening was scored as 1 point; diffuse stromal edema was scored as 2 points; diffuse stromal edema with small blisters was scored as 3 points; bullous keratopathy was scored as 4 points. (3) New vessels generation: no neovascularization was scored as 0 point; neovascularization appearing at the surrounding area of the engraftment bed was scored as 1 point; neovascularization appearing at the peripheral area of the graft was scored as 2 points; neovascularization appearing at the central area of the cornea was scored as 3 points; full cornea graft neovascularization was scored as 4 points. The sum of the three scoring criteria: the corneal opacity, edema, and vascularization represent the RI. Corneal rejection occurs when RI was scored as greater than 6. The time of appearance of corneal rejection in each group was recorded.

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**Figure 1.** Changes of the corneal graft in group B rats, 5 days and 14 days post operation. A: Five days post operation, graft edema, cornea opacity and neovascularization; B: 14 days post operation, graft edema and neovascularization.



**Figure 2.** Changes of the corneal graft in group C rats, 5 days and 14 days post operation. A: Five days post operation, graft edema, cornea opacity and neovascularization. B: 14 days post operation, transparent graft and new blood vessels receded.

### Statistical analysis

All statistical analyses were performed using SPSS 13.0 software. All measurement data were presented as  $\bar{x} \pm s$  (mean  $\pm$  s.d.) and comparison between groups were performed using two-way repeated measurement ANOVA.  $P < 0.05$  was considered statistically significant.

### Results

#### *Clinical observation of cornea allograft rejection*

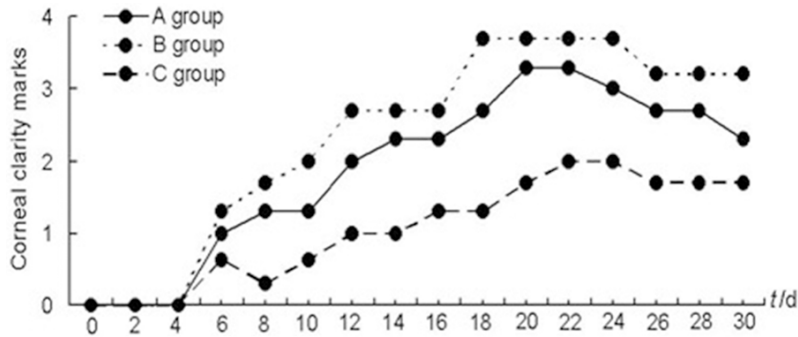
The penetrating corneal transplants in the rats belonging to the three groups were successful. Five days post operation, group A and B rats showed a cornea opacity score of 1~2 points as well as for graft edema. Two weeks post opera-

tion, the cornea opacity score was 2~3 points, graft edema was 2~3 points, and a large number of new blood vessels formation could be seen (**Figure 1**). Five days post operation, group C rats showed a cornea opacity score of 1~2 points and graft edema 1~2 points. Two weeks post operation, the grafts became transparent, without edema, and the neovascularization was reduced (**Figure 2**). Fundus examination showed that the retina was in its normal position and presenting a ruddy color. All rats showed no lens injury during the observation period. No eye infections or no complications of the retina and vitreous were observed.

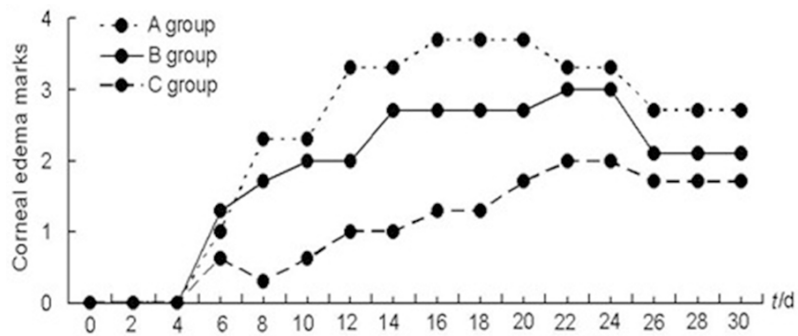
#### *Changes in corneal grafts*

The RI ratings of the corneal grafts regarding transparency, edema and neovascularization, and the total RI rating of the three transplant

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**Figure 3.** Changes in the degrees of transparency in the corneal grafts at different time points.



**Figure 4.** Changes in the degrees of corneal edema in the corneal grafts at different time points.

groups were as follows: all RI ratings in group B were significantly increased compared to group A ( $P < 0.05$ ), as shown in **Figures 3-6**. At 5 days post operation, neovascularization in group A, B, and C were limited to the corneal limbus. At 15 days after operation, the neovascularization in group B showed no statistical difference ( $P > 0.05$ ) compared to group A, while the neovascularization in group C was significantly reduced ( $P < 0.05$ ).

### Discussion

Previous studies showed that corneal allograft rejection is a complex process involving many factors, in which cell immunity plays a major role [5]. Although under the treatment of local or systemic immunosuppressive agents, the incidence of immunological rejection after corneal transplantation remains high. Hence, reducing the occurrence of postoperative immune rejection and avoiding secondary corneal transplantation are both factors of extreme importance. Currently, drugs still represent the

first choice for the treatment of immunological rejection after high-risk corneal transplantation. However, the in-depth studies regarding the efficacy of the current immunosuppressive agents revealed that more and more adverse reactions occur at a local or systemic level. Many researchers made great efforts to reduce the drug adverse reactions through different routes of drug administration, drug preparation types and combination usage of drugs. In recent years, clinical studies confirmed that corneal allograft rejections are closely related to factors such as surgical procedures, the size of the implant, the degree of corneal neovascularization, the severity of damage in the cornea, the degree of infections, and the numbers of operation per-

formed, just to cite the most important and common. Ti SE *et al.* [6] reported the outcomes of 92 cases of corneal keratoplasty due to progress keratitis. Seventy eight patients resulted positive on pathogen culture. Among them, 31 patients presented fungal infections, mainly by *Fusarium*. Seventy four patients showed a good therapeutic outcome after the first surgery. No differences in the cure rates of fungal and bacterial infections 1 year after the operation, while the treatment resulted unsuccessful in 15 patients, 11 of them due to the recurrence of fungal infections. Therefore, fungal keratitis after penetrating keratoplasty represents one of the main problems for a successful keratoplasty. Garcia *et al.* [7] reported that intracorneal injection of 0.1 ml of Amphotericin B (0.5 ug/ml) is a convenient and effective way to treat recurrent fungal keratitis and endophthalmitis after penetrating keratoplasty. The antibacterial mechanism of Trichostatin is similar to that of Amphotericin B. Basically, they both bind to the sterols on the fungal cell membrane, forming micropores on



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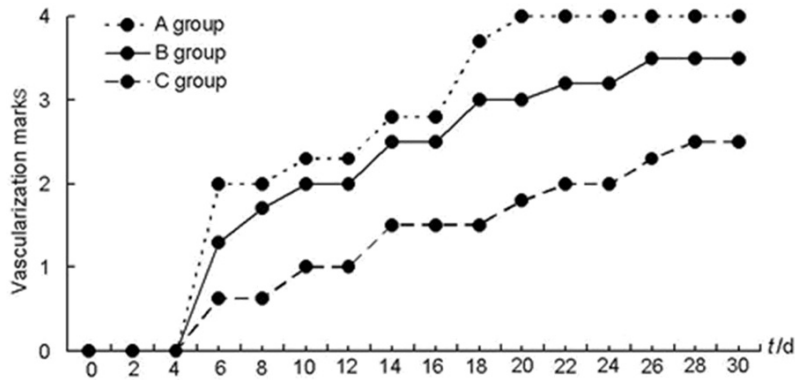


Figure 5. Changes in the degrees of neovascularization in the corneal grafts at different time points.

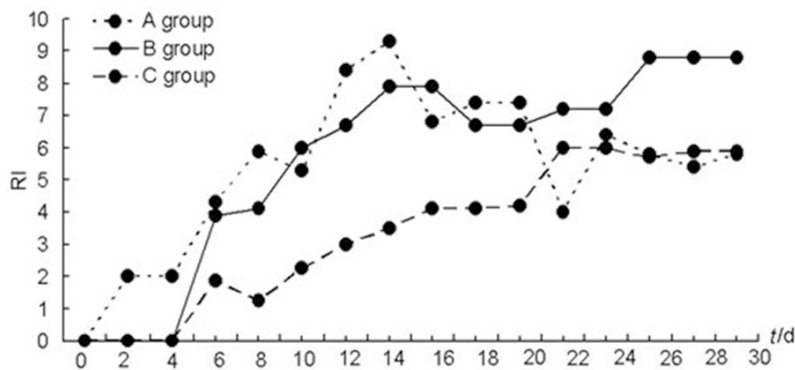


Figure 6. Changes of RI of the corneal grafts at different time points.

the membrane. This bind and micropore formation alters the permeability of the cell membrane, resulting in a leakage of the essential nutrients and amino acids from the fungal cells, destroying their normal metabolism and inhibiting their growth.

Corneal transplant induces neovascularization into the surface and in the stromal layer of the corneal bed of the recipient, due to surgical trauma. Previous studies showed that neovascularization is actually the main risk factor of corneal transplant rejection [8]. In this study, the retaining sutures and the exposing suture nodes produced the effect of inducing corneal neovascularization. Therefore this animal model possesses, to some extent, the characteristics of high risk transplant. Currently, the topical medications clinically used for the suppression of immunological rejection after high-risk corneal keratoplasty are glucocorticoids, cyclosporine A (CsA), and FK506. These medications can improve the success rate of trans-

plants and the long-term survival rate of the grafted cornea, but rejections could not be avoided in some patients. CsA and FK506 inhibit calcineurin, block the expression and transcription of cytokines such as IL-2, inhibit T cell activation at early stage, and inhibit T cells G0 to G1 transition. Trichostatin is a polyene antibiotics extracted from the culture media of *Streptothrix*. It possesses strong inhibitory effect on the growth of various fungi and protozoa, as well as both anti-fungal and anti-tumor effects. Trichostatin can bind to sterols on the fungal cell membrane, and destroy fungal normal metabolism. To avoid the potential risks of side effects and the whole body immunosuppression resulted from systemic medication, we used Trichostatin as a topic medication, directly dropping it

into the eye. The results of this study showed that the three groups of rats received a successful keratoplasty. Two weeks after the operation, group C rats treated with Trichostatin showed no edema, and neovascularization was reduced. Rejection in group C was significantly lower than that of group A or B. The degrees of transparency, edema, angiogenesis of the corneal grafts, and the general RI scores of the group C rats were significantly reduced. These results showed that Trichostatin possessed inhibitory effects on the rejection reactions, such as corneal edema, reduced corneal opacity and reduced proliferation of the neovascularization after penetrating keratoplasty due to fungal infection.

Immunological rejection after high-risk corneal transplantation is the main reason of operation failure. Thus, it is of utmost importance to solve this serious problem. Trichostatin eye drops showed good prospects in the inhibition of immune rejection after high-risk corneal trans-

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plantation. Further studies should be performed to optimize the drug concentration, dosage and the long-term effect after simultaneous use with other immunosuppressants, in the treatment of the immune rejection after allergenic transplantation.

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### Disclosure of conflict of interest

None.

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