

# Evaluation of corneal graft survival in mice model

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## Abstract

• **AIM:** To investigate the characteristics and criterion of graft rejection in mice model.

• **METHODS:** C57BL/6 or BALB/c mice corneal grafts were grafted onto BALB/c hosts. Each group was divided into two subgroups according to the corneal opacity scores 12d after transplantation. The characteristics of opacity and neovascularization were observed. Mice of the 12<sup>th</sup>, 50<sup>th</sup> day after transplantation, the grafts biopsy of mice in allogeneic group 1, which opacity score exceed 3, were prepared for histological observation and those restore transparent were endothelial stained.

• **RESULTS:** There was no difference of corneal opacity score on the 7<sup>th</sup> and 12<sup>th</sup> day after operation; the histological results had no disparity between syngeneic group and allogeneic group. On the 12<sup>th</sup> day after surgery, the turbidity curve was apparent in grafts with opacity score <2. Mononuclear cells were shown in grafts with opacity score reached 3 in allogeneic group 1. Different rejection performance was observed in tissue sections on the 50<sup>th</sup> day after surgery.

• **CONCLUSION:** Grafts, opacity score exceeds 3 from the 7<sup>th</sup> to the 12<sup>th</sup> day after operation could not be judged as a rejection. We should pay more attention to the variation of grafts opacity since 12d after corneal transplantation.

• **KEYWORDS:** corneal transplantation; graft survival; experimental study

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## INTRODUCTION

Since the first successful penetrating keratoplasty (PKP) was reported by Edward Zirm in 1906, corneal transplant has become the oldest, most successful and common form of tissue transplantation [1,2]. When corneal grafts are placed into an avascular recipient bed (so-called normal-risk keratoplasty), 2-year graft survival rates reach 90% under cover of topical steroids, even without HLA-matching[1,3,4]. Although corneal transplantation is one of the most common tissue transplantations and is known to have a high graft acceptance rate, irreversible rejection is clearly the important cause of graft failure, despite the long-held view that the cornea is an immune-privileged tissue in an immune-privileged site[4-6].

In the study of corneal transplant rejection, mouse model has been widely used [1,7-9]. Mouse cornea is relatively smaller; its pupil is only 3.0mm to 3.5mm in diameter. Compared to other experimental animals, it is more difficult to have a corneal transplantation in their eyes. Mouse is one of the most widely used experimental animals in medical research, as the model of corneal transplant rejection, mice have their unique advantages. The opaque level is often used as the exclusion criteria at the mouse corneal transplantation model; but the occurrence of corneal opacity is not the determinant sign of a graft rejection. The transparent corneal grafts could also be observed after the corneal graft rejection. The aim of the present research was to observe the characteristics of corneal rejection at different times after transplantation and the criterion of corneal graft rejection in mice.

## MATERIALS AND METHODS

### Materials

**Mice and anesthesia** In mouse keratoplasty model, female BALB/c mice (H-2d,  $n=60$ ) at the age of 6-8 weeks (18-22g) were used as recipient, and female C57BL/6 mice (H-2b,  $n=15$ ) at the same age as graft donor both provided by the Animal Center of the General Hospital of Chinese People's Liberation Army (Beijing, China). For syngeneic transplantations, 30 BALB/c mice were both donors and

**Table 1 Clinical scoring system for corneal grafts**

Score	Clinical appearance
Opacity	
0	Clear cornea graft
1	Minimal, superficial (not involving stroma) opacity, pupil margin and iris texture is readily visible
2	Minimal to moderate opacity including corneal stroma, pupil margin and iris texture is visible
3	Moderate stromal opacity, only pupil margin visible
4	Intense stromal opacity, only outline of pupil is visible
5	Severe stromal opacity, anterior chamber is invisible
Neovascularization	
0	No neovascularization
1	Neovascularization around the recipient graft beds only
2	Neovascularization around the peripheral graft
3	Middle and peripheral part of graft were vascularized
4	Entire cornea vascularized

recipients (syngeneic group), among syngeneic groups, those cornea opacity score  $\leq 2$  were defined as syngeneic group 1,  $\geq 3$  as syngeneic group 2; other 30 BALB/c mice accepted allogeneic corneal transplantation (allogeneic group) among syngeneic groups, those cornea opacity score  $\leq 2$  were defined as allogeneic group 1,  $\geq 3$  as allogeneic group 2. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and this project was approved by the institutional ethics committee. Anesthesia was administered intraperitoneally by ketamine/Sumianxin solution at a dose of 5.0mg/kg and 2.5mg/kg, respectively.

## Methods

**Corneal transplantation** Before transplantation, compound tropicamide eye drops (Santen, Japan) were used to dilate pupils and oxybuprocaine hydrochloride eye drops (Santen, Japan) for surface anesthesia. Penetrating keratoplasty in mice has been described in previous studies [10,11]. Briefly, C57BL/6 or BALB/c donor corneal grafts were excised by removing a central 2-mm corneal button using a 2.0-mm mouse trephine and cut with a sharp-tip vannis scissor (66 vision, Suzhou, China).

Under anesthesia, a 2-mm graft bed of the right eye in a recipient was prepared by trephining the central cornea. Then a donor corneal button was immediately planted into the graft bed in place with 8 interrupted sutures (11-0 nylon, Tyco Healthcare Group, USA) under TOPCON OMS300 surgical microscope (Topcon, Japan). While in allograft, grafts were sutured into the graft bed after rotating 180 degrees in orthotopic position.

Before suturing the eyelids with 10-0 nylon suture (Tyco Healthcare Group, USA), antibiotic ointment (Hlortetracycline, Jinan, China) was applied on the corneal surface. Then eyelid suture was removed 24h later, and the grafts with technical difficulties including hyphema, cataract, infection, and loss of

anterior chamber were excluded from further consideration. Corneal sutures were removed after 7d, and grafts were examined every 2d until 50d after transplantation under slit lamp microscopy (66vision, Suzhou, China).

**Evaluation of graft survival** According to the Sonoda and Streilein [12] and our preliminary experimental results, corneal grafts were examined everyday in the first 14d (expect day 1) under an operating microscope, and every 2d from 15d-50d postoperatively. Corneal graft score was reported by 2 independent observers according to the same clinical scoring system for corneal graft (Table 1). In this study, a graft rejection was determined by the opacity and neovascularization in accordance with previous studies [7,10,12]. A graft with opacity of 3 or greater and neovascularization score of 2 or greater after suture removal was considered as graft rejection.

**Histological evaluation of corneal grafts** On day 12 and 50 after corneal transplantation and when grafts express rejection in allogeneic group 1, three mice of each group were sacrificed by dislocation and used for histological examination. The grafted eye was enucleated, washed in running water, fixed in 4% paraformaldehyde for 6h, embedded in paraffin. The 5-mm slices were prepared (Leica, Germany) and then routinely stained by hematoxylin and eosin for light microscopy examination.

**Endothelial staining** Mice with transparent corneas of syngeneic group and allogeneic group were killed by dislocation and all corneal tissues were cut radically along the limbus. Endothelial staining was done by using 0.25% alizarin red and 0.25% blue cone (Chemical Reagent Co, Beijing, China) for 150s with the flat endothelial surface on top. The staining was examined through light microscope after washing 3 times.

**Statistical Analysis** An analysis was performed by using SPSS 13.0. Analysis of variance was used for compare

corneal opacity and neovascularization scores. A *P* value less than 0.05 was considered of statistical significance.

**RESULTS**

**Clinical Observation of Corneal Grafts** Three mice (5%) were excluded due to complications including cataract in 2 cases and iris incarceration in 1 case. The number of successful cases was shown in Table 2.

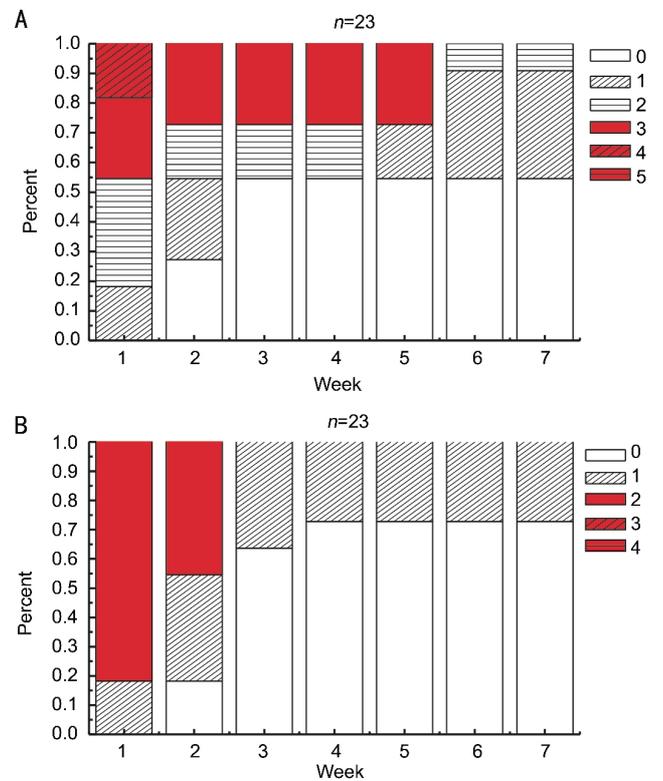
**Changes of Graft Transparency**

**Grafts in syngeneic group** The corneal transparency decreased gradually before sutures removed. On day 7 after transplantation, grafts of an opacity score  $\geq 3$  were 38% and of a neovascularization score  $\geq 2$  were 83%. After suture removal, 79% grafts became clearer and the opacity score reduced to 2 or less and the neovascularization decreased quickly within 12d after operation. Grafts of opacity score  $\geq 3$  were 21%. All grafts with opacity score more than 3 on days 12 were that opacity exceeded 3 on day 7 postoperatively and did not become clear. The largest proportion of higher opacity and neovascularization scores appeared in the first week postoperatively, and then decreased gradually with time. The different opacity and neovascularization in syngeneic group was shown in Figure 1. There were significant differences in the opacity percentage of grafts ( $F=37.390, P<0.05$ ). Except day 5-7, corneal opacity scores were significantly different between syngeneic group 1 and syngeneic group 2. There was significant difference in corneal opacity score between allogeneic group 1 and allogeneic group 2 before day 21 postoperatively (Figure 2).

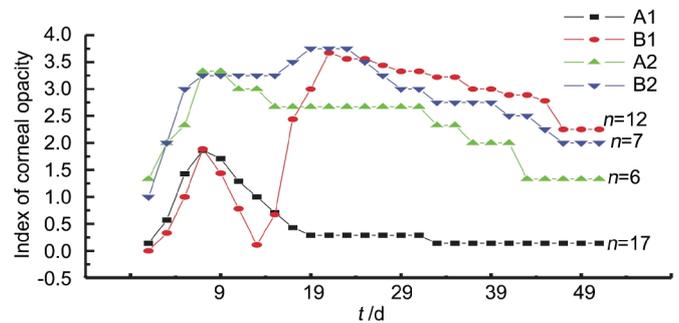
**Grafts in allogeneic group** The corneal transparency decreased gradually before the suture removal. On day 7 postoperatively, the grafts of an opacity score  $\geq 3$  was 59% and of a neovascularization score  $\geq 2$  was 86%. Between days 7 and 12, 75% grafts became clearer and their opacity score reduced to 2 or lower, and the density of neovascularization decreased in varying degrees. Grafts of opacity score  $\geq 3$  were 25% on the 12<sup>th</sup> day after transplantation. Neovascularization increased and invaded the graft bed after 14d. Different levels of edema and opacity with worse turbidity occurred around the neovascularization site and the grafts became completely opaque gradually. The larger proportion of higher grafts opacity and neovascularization scores appeared in the first and second week, respectively. This proportion increased since the third week and reached its peak in the fourth week. Four weeks later, the grafts of higher opacity score decreased gradually at a higher level. The different turbidity and neovascularization in allogeneic group was shown in Figure 3. There were significant differences in the corneal neovascularization scores of grafts ( $F=16.657, P<0.05$ ). Except day 11-15, corneal neovascularization had significant differences between syngeneic group 1 and syngeneic group 2. There were significant differences in corneal opacity score between

**Table 2 Animal numbers of each group**

Group	Syngeneic group		Allogeneic group	
	1	2	1	2
<i>n</i>	23	6	21	7
Total number	29		28	



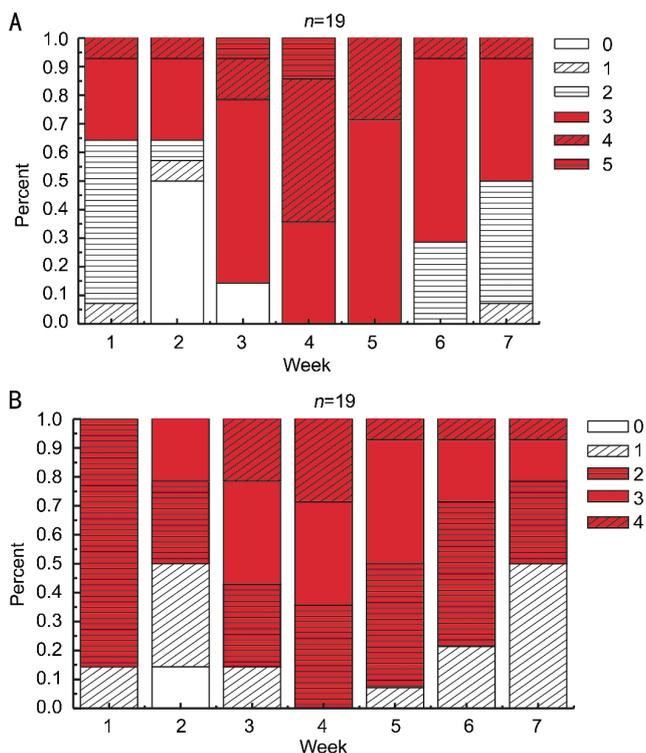
**Figure 1 Graft percentage of different turbidity (A) and neovascularization (B) in syngeneic group.**



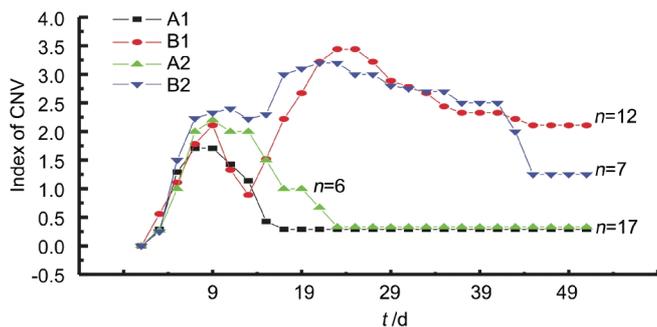
**Figure 2 Trends of corneal opacity score in each group** A1: Syngeneic group 1; A2: Syngeneic group 2; B1: Allogeneic group 1; B2: Allogeneic group 2.

allogeneic group 1 and allogeneic group 2 expect for day 11-15 (Figure 4). At the end of observation, persistent opacity was found without obvious edema (*n*=3) (Figure 5A); Grafts returned clarity without edema, just left several point opacities in stromal with opacity score  $\leq 2$  (*n*=2) (Figure 5B). Grafts showed a bubble shape without neovascularization and parts of grafts showed cone shape (*n*=12) (Figure 5C).

**Opacity Scores of Graft** There was no significant difference in the proportion of corneal opacity score between syngeneic group and allogeneic group on the 7<sup>th</sup> ( $\chi^2=0.643, P=0.423$ ) and the 12<sup>th</sup> day ( $\chi^2=0.018, P=0.895$ ) after surgery (Tables 3, 4).



**Figure 3** Graft percentage of different turbidity (A) and neovascularization (B) in allogeneic group.



**Figure 4** Trends of corneal neovascularization score in each group A1: Syngeneic group 1; A2: Syngeneic group 2; B1: Allogeneic group 1; B2: Allogeneic group 2.

**Table 3** Corneal opacity scores of each group on the 7<sup>th</sup> day

Corneal opacity scores	Syngeneic group (n)	Allogeneic group (n)
≤2	18	14
≥3	11	14

**Table 4** Corneal opacity score of each group on the 12<sup>th</sup> day

Corneal opacity scores	Syngeneic group (n)	Allogeneic group (n)
≤2	23	21
≥3	6	7

**Histology Results** On day 12 postoperatively, the histology findings in syngeneic group were similar to Allogeneic group. A small amount of inflammatory cells were found in all grafts regardless of opacity scores. The stromal of grafts with opacity score ≥3 were thicker than score ≤2 (Figure 5D, E, F, G). On day 50 postoperatively, graft structures were similarly normal in syngeneic group (Figure 5H, I). No inflammatory cell was found in allogeneic group. In grafts

with persistent opacity, epithelium was thinner and stromal was thicker than normal ones (Figure 5J). In those grafts restoring clearer, the corneal tissue had continuous endothelial cell layer and similar with normal corneas, and thinner than normal ones (Figure 5K). In grafts showing a bubble shape, the epithelium and stromal layer were thinner obviously, and layered phenomenon could be found in the stromal (Figure 5L).

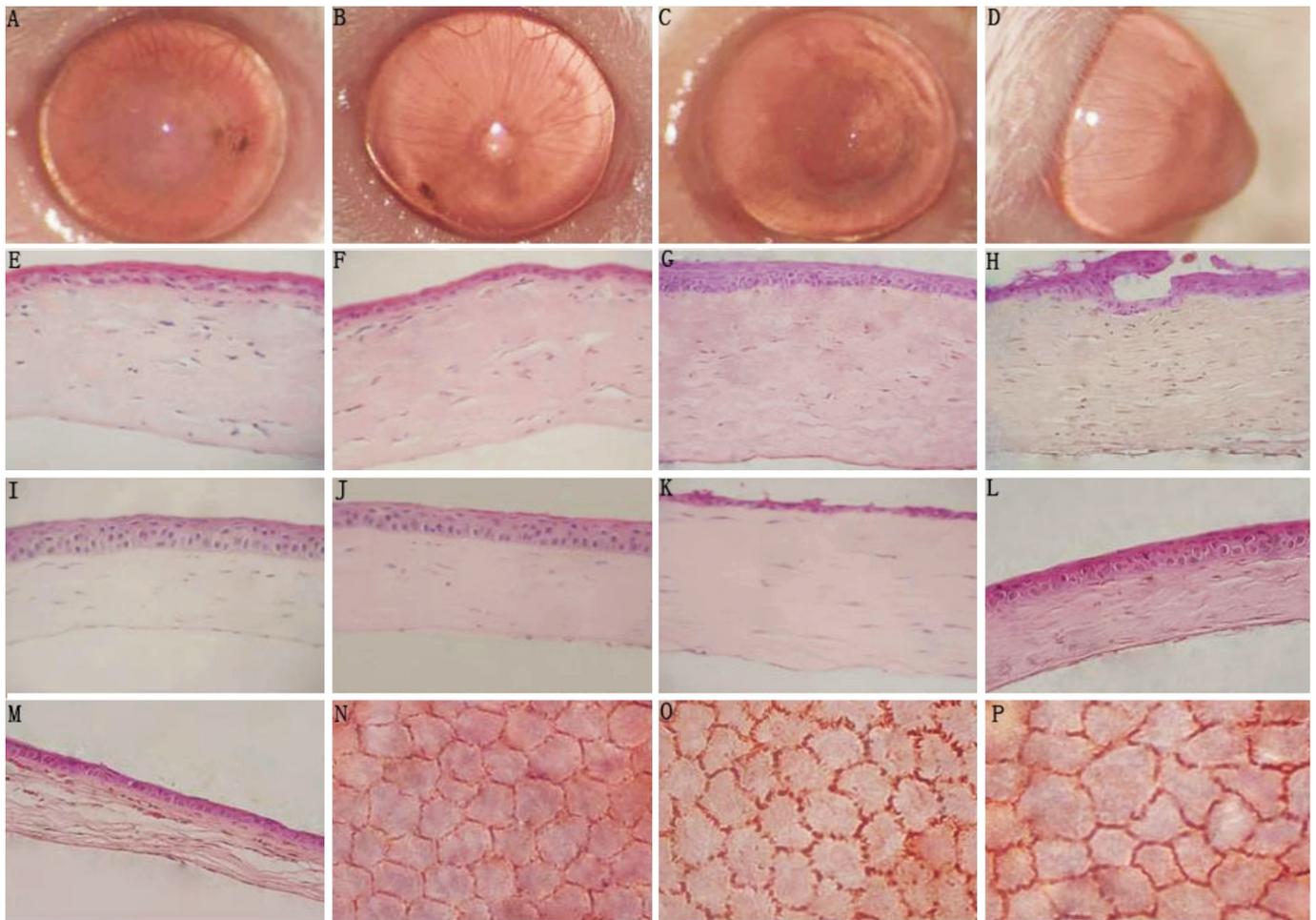
**Results of Endothelial Staining** The endothelial cells showed the same size and arranged neatly with hexagonal shape in transparent grafts of syngeneic group (Figure 5N), just like normal (Figure 5O). In those grafts restoring transparency of allogeneic group, endothelial cells showed unequal size and arranged disorderly with various shapes (Figure 5P).

**DISCUSSION**

Early studies utilized rabbits and, more recently, sheep, but the availability of a large range of reagents for rodents has permitted more mechanistic studies on rats and mice [13-15]. With the improvement of surgical instruments and suture material advances, corneal transplants became possible in mice [1,7-9,16,17]. In 1990, She *et al* [16] first reported the orthotopic corneal transplants in mice. The fundamental purpose of this research was to study the mechanism of corneal rejection and find the methods to inhibit it. Therefore, how to define and determine the corneal graft rejection was really important. In clinical and experimental reports, the corneal graft rejection usually related to corneal opacities. Thus, the diagnosis of graft rejection is based on loss of graft transparency [7,18-20].

We know that the presence of preexisting blood vessels is a strong risk factor for corneal graft immune rejection. Normal cornea is avascular, which is an essential element of corneal transparency [21]. In our study, we found that neovascularization emerged on day 3 and extended to the junction of graft bed on day 6 and 7 postoperatively. The filling of new blood vessels reduced at varying degrees after suture removal which would not disappear completely. The neovascularization might be induced by receptors, but not inflammation-specific stimulated by the suture in place. After that, corneal edema and opacity became obvious and accompanied by re-filling of new blood vessels when corneal grafts rejected. Therefore, we believed that neovascularization at the border of graft is a prerequisite of corneal transplantation rejection regardless of its invading degree.

The main purpose of corneal transplantation research is to study the rejection mechanism and the methods to treat and prevent rejection after surgery. Various factors can contribute to opacity or reduced clarity of cornea including cellular infiltration, new vessel growth, thickening and irregularity of cornea and edema. However, the mouse model has its own characteristics. In present experiment, corneal opacity



**Figure 5 Clinical evaluation, histology results and endothelial staining of mouse corneal graft** 1) Clinical evaluation of mouse corneal graft on day 50 after transplantation in allogeneic group (A, B, C, D). A: Persistent opacity without obvious edema; B: Point-like, thread-like opacities in stroma without edema; C, D: Grafts showed cone shape. 2) Histology results of grafts on day 8 postoperatively (E, F, G, H). E: Grafts of opacity score 2 in syngeneic group; F: Grafts of opacity score 2 in allogeneic group; G: Grafts of opacity score 3 in syngeneic group; H: Grafts of opacity score 3 in allogeneic group; I: Histology results of normal cornea; J: Histology results of grafts on day 50 postoperatively in syngeneic group. Histology results of grafts on day 50 postoperatively in allogeneic group (K, L, M). K: Grafts with persistent opacity; L: Grafts regain clear; M: Grafts shown a bubble shape. 3) Results of endothelial staining (N, O, P); N: Endothelial staining of normal cornea; O: Grafts of syngeneic group, arranged with normal shape; P: Grafts of allogeneic group, cells arranged disorderly with various shape.

changed obviously both in allograft and autologous transplantation. Before sutures holding the graft in place were removed, edema of corneal epithelial increased and corneal transparency decreased gradually after surgery. The grafts performance of allograft is similar with those of autologous transplantation before suture removal. The corneal transparency decreased gradually.

The corneal density of neovascularization decreased at varying degrees. Fourteen days later, the extent of neovascularization increased again and invaded the cornea graft; different degrees of edema and opacity formed with heavier turbidity around the neovascularization site, and the graft completely became opaque gradually. The grafts opacity score  $\geq 3$  were 25% on the 12<sup>th</sup> day after transplantation, and keeping continuous opacity.

Many studies have demonstrated that the declining corneal transplantation is related to temporary loss of corneal

endothelial function around the 7<sup>th</sup> day after transplantation<sup>[22-24]</sup>. However, we hypothesize that the early transient peak in corneal opacity following closely suture removal is likely to represent non-specific inflammatory irritation (otherwise known as the innate immune responses) caused by surgery and suture rather than immunological rejection, and the suture may be the main factor. Plsková *et al*<sup>[7]</sup> compared the rejection between continuous and interrupted suture, and found that the corneal graft with interrupted sutures occurred rejection earlier than those with continuous suture. Interrupted suture was used in the present study and it was found that histology results in allograft were similar with those of autologous transplantation 7d after transplantation. The allograft grafts that meet the corneal opacity and neovascularization rejection score were not rejected after 7d. There were no differences of opacity score among groups on day 7 and 12 after corneal transplantation. Heavier opacity

may be related to endothelial injury and suture stimulation caused by surgery as a result of repair response. More attention is required to observe grafts opacity around day 7 after transplantation, especially for those with opacity score reached 3. Grafts could be removed if the opacity score remains greater than 3. According to our results, rejection has appeared in some grafts on the 14<sup>th</sup> day after corneal transplantation. If the opacity does not improve 12d later, it is essential to decide whether the corneal graft rejection has occurred or not.

Allogeneic corneal grafts rejection in rats appeared in 95%-100% cases in previous studies [12,25,26]. In this study, rejection rate was 100%. The following outcomes were found: 1) entire cornea vascularized and opacity without edema; 2) grafts showed a bubble shape without neovascularization, and the grafts showed cone; 3) opacity graft returned clear without edema, but point opacities still existed in corneal stromal. For the first two outcomes, it was easier to assess a rejection. But for the last one, it was easier to misjudge a rejection.

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