·Basic Research·

Experimental study on cervical lymph nodes removal enhance allograft survival in alkali-burned cornea

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Abstract

• AIM: To explore the inhibitive effects of cervical lymphadenectomy on keratoplasy after alkaline burns.

• METHODS: The Wistar rats' corneas were transplanted into Sprague-Dawley (SD) rats' eyes which were randomly divided into 4 groups: group A (control group); group B, the cervical lymphadenectomy group; group C, corneal transplantation after the alkali burn injury; group D, cervical lymphadenectomy following group C. Out of 6 rats in each group, the cornea of one rat was used for macrophage immunohistochemistry at day 14 after the transplantation, and the remaining 5 rats were used for studying corneal immune rejection with a slit lamp. The time when allograft rejection occurred was recorded and mean survival times (MST) were compared among the groups.

• RESULTS: Compared with the MST of group A (10.40± 1.14 days),the MST of group B(46.30± 9.46 days) was significantly longer (P<0.05). MST of grafts between group C (7.00± 1.58 days) and group D (15.00± 3.39 days) was also significant (P< 0.05). At 14th day after the transplantation, there was no CD_{68} immunoreactivity in the graft of group B, and CD_{68} proteins were expressed to some extent in the grafts of group A and D. However, in the graft of group C, the expression of proteins was dramatically up-regulated.

• CONCLUSION: Cervical lymphadenectomy therapy has a significant effect in preventing corneal allograft rejection in normal and alkali burned corneal beds.

• KEYWORDS: alkaline burns; corneal transplantation; allograft rejection; lymphadenectomy

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INTRODUCTION

Alkali injuries of the eye often cause extensive damage to cornea and anterior segment resulting in permanent visual impairment. It is reported that the survival rate of corneal grafts in the alkali-burned cornea recipient is no more than 10%, mainly due to the severe inflammation and vascularization [1]. Studies by Feng *et al* [2] also showed that among all ocular diseases receiving keratoplasy, ocular chemical burns had the worst prognosis. Recently, Yamagami *et al* [3] has provided evidence that cervical lymph nodes play an important role in corneal alloimmunization and succeeded to inhibit allograft rejection in a model of suture-induced high risk keratoplasty by cervical lymphadenectomy. However, whether such a method is helpful to inhibit allograft rejection in the alkali-burned cornea still remains unknown.

MATERIALS AND METHODS

Animals Twenty-four SD rats were used as recipients, and 12 Wistar rats as donors for corneal transplantation. Animals weighing 150-200g, female, 1-2 months old each, obtained from the Animal Care Center of Sun Yat-sen University, China. The recipients were randomly divided into 4 groups: Group A was the normal corneal transplantation group (control group); Group B, the cervical lymphadenectomy group; Group C, corneal transplantation after the alkali burn injury; Group D, cervical lymphadenectomy following group C. Among the 6 rats in each group, 5 were used for observation of corneal allograft rejection by the slit lamp and the remaining 1 for CD_{68} immunohistochemistry. The experimental conditions used in the study conformed to good laboratory practices (National Research Council USA 1996) and all animals used in the study was handled in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research.

Rat Alkali Injured Model Rats were anesthetized with peritoneal administration of Chloral Hydrate (300mg/kg). A filter paper disc, 3mm in diameter, was dripped with 1mol/L NaOH solution for 20 seconds, then it was placed on the central cornea of the right eye for 30 seconds, then injured eyes were rinsed with sterile physiologic saline (9g/L NaCl, 10mL).

Orthotopic Corneal Transplantation The procedure was adapted from the technique described previously by Slegers[4]. The rats were anesthetized and 10g/L pilocarpine was used for 20 minutes before the operation. Donor corneas (Wister) 2.75mm in diameter were removed and placed in Optisol solution. The recipient right cornea was marked with 2.5mm trephine and excised under the operating microscope. Then, the donor graft was sutured into the recipient bed with six interrupted sutures (10-0 nylon). In group C and D, corneal transplantation was performed 21 days after corneal alkaline burn.

Surgical Removal of Cervical Lymph Nodes After corneal transplantation in group B and D, hair of rat neck was shaved, then a small incision was made in the neck skin under operating microscope. Cervical lymph nodes (CLNs, Figure 1), including superficial lymph nodes (3-4 lymph nodes usually), submandibular lymph nodes, anterior CLNs, thyroid lymph nodes, anterior jugular lymph nodes, and superficial lateral CLNs, were excised step by step, but carotid artery and cervical vein were not spared. At last, incision was closed by interrupt sutures (8-0 nylon).

Observation under a Slit Lamp Microscope The graft was examined by the slit lamp microscope everyday after transplantation in 2 weeks, then twice a week till 60 days. Clinical appearances of each graft were scored using following three criteria: graft opacity, graft edema and graft vascularization. The scoring scheme were based on Hollands' control [6] and grafts with scoring 6 or higher were recorded as rejected. Eyes with the complications like postoperative cataract, infection, or hyphema were excluded from the study and replaced by new animals.

Immunohistochemistry One rat was sacrificed randomly in each group at 14th day after corneal transplantation. Grafts were fixed in 100mL/L neutral buffered formalin for 24 hours, then, embedded in paraffin, serially sectioned for 4μm in thickness for macrophage immunohistochemistry.

Goat anti rat CD_{68} monoclonal antibody (a special antibody for macrophages) was applied as first antibody and biotin marked rabbit anti goat immunoglobulin as the secondary antibody. The slides were visualized for peroxidase activity with diaminobenzidine (DAB) and counterstained with hematoxylin.

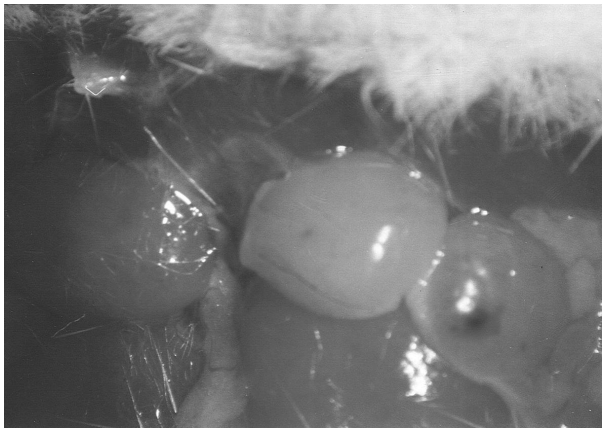

Figure 1 Cervical lymph nodes in rats. Magnification ×10

Table 1 Mean survival time of grafts in every group

(mean±SD)

Group	Eyes	MST(day)
A	5	10.40±1.14
B	5	46.30±9.46[a,c]
C	5	7.00±1.58[a]
D	5	15.00±3.39[a,c]

[a]P <0.05 *vs* group A; [c]P<0.05 *vs* group C

Statistical Analysis The scores and MST were compared among the four groups respectively. Values are presented as mean ±SD. The significance of differences between two groups were analyzed by paired Student's t-test (SPSS 10.0 statistical software). Differences were accepted as significant at P<0.05.

RESULTS

Effect of Lymphadenectomy on Corneal Allograft Survival At 7th day after corneal transplantation, grafts in group C were being rejected by showing considerable corneal new blood invasion. At 14th day after transplantation, partial grafts in both group A and D began to show rejection, while no graft failed in group B (Figure 2). As compared with group A (10.40±1.14 days), graft survival time in group B (46.30 ±9.46 days) was dramatically prolonged (P<0.05). The difference in mean survival time (MST) of grafts between group C (7.00±1.58 days) and group D (15.00±3.39 days) was also significant (P<0.05, Table 1). At 14th day after corneal transplantation, corneal rejection parameters in groups D, including graft opacity, graft edema and graft vascularization, reject index (RI), were much lower than those in group C (P<0.05, Table 2). It suggested that lymphadenectomy therapies could effectively prevent corneal allograft rejection, no matter in normal-risk beds or in alkali induced high-risk beds.

Immunohistochemistry In group A and D, CD_{68} proteins were weakly expressed in the grafts. In group C, grafts showed edema and corneal hypothallus was obviously thickened with disordered collagen fibers and strong

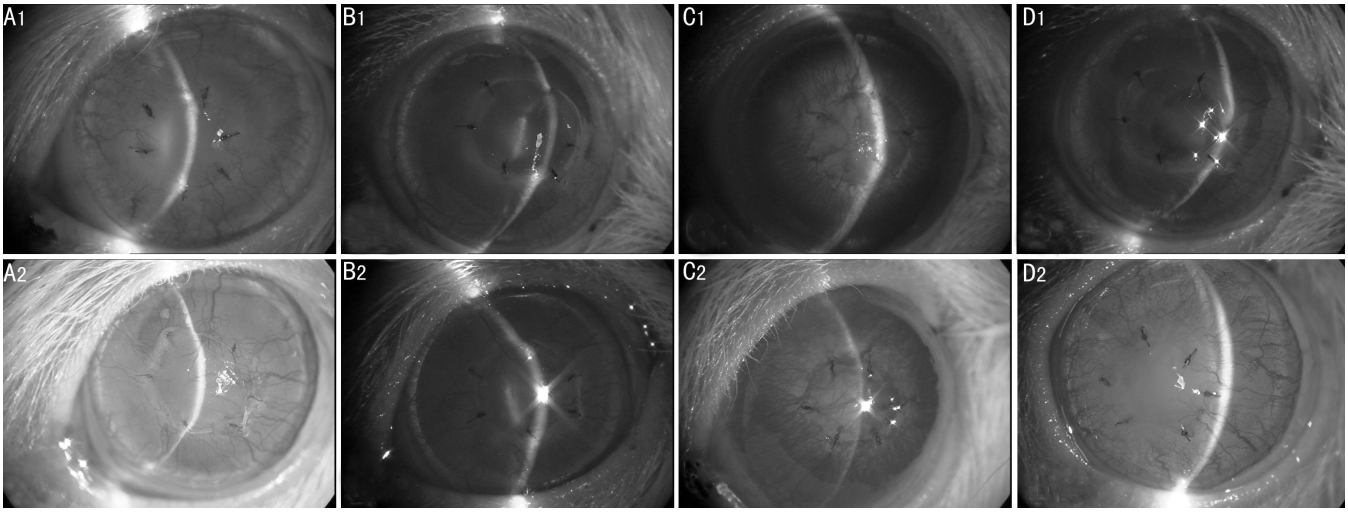

Figure 2 Observation under a Slit Lamp Microscope after corneal transplantation In group A, at 7th day after transplantation, the grafts remains transparent, but at 14th day after transplantation, partial of grafts failed due to corneal vascularization, edema or opacity (A). In group B, all grafts survived both at 7th and 14th day after corneal transplantation (B). However, in group C, almost all grafts failed at 7th day after transplantation, and at 14th day after transplantation, new blood vessels were full of the grafts (C). In group D, at 7th day after transplantation, the grafts were transparent to see the pupils, at 14th day after transplantation, most grafts still survived although there were some blood vessels extending from limbus to the grafts (D). (Footnote1: 7 days after corneal transplantation; Footnote 2: 14 days after corneal transplantation. **Magnification ×25**

Table 2 Clinical parameters in every group at 14th day after transplantation (mean±SD)

Group	Eyes	Graft opacity	Graft edema	Graft vascularization	RI
A	5	2.80±0.84	2.80±0.84	2.20±0.45	7.80±0.84
B	5	1.10±0.31[a,c]	1.30±0.95[a,c]	1.40±0.52[a,c]	3.60±1.17[a,c]
C	5	3.60±0.55[a]	3.40±0.55[a]	3.80±0.45[a]	10.80±0.84[a]
D	5	2.60±0.55[a,c]	1.60±0.89[a,c]	2.80±0.84[a,c]	7.00±1.22[a,c]

[a]$P < 0.05$ *vs* group A; [c]$P < 0.05$ *vs* group C

immunoreactivity of CD_{68}, suggesting a large number of macrophages infiltration at that time point. In addition, posterior elastic and endothelium layer were nearly disappeared. In contrast, the grafts in group B were nearly intact with no CD_{68} immunoreactivity (Figure 3).

DISCUSSION

Alkali injuries of the eye represent one of the most serious forms of eye trauma and may cause extensive damage to ocular surface including cornea, and anterior segment, resulting in irreversible vision loss. Although corneal transplantation is the most commonly and successfully performed tissue transplantation, the management of alkaline cornea burns has been particularly challenging because some of the classic forms of tissue (e.g., HLA) matching and immunosuppressive therapy have shown only little efficacy in promoting graft survival .

It is well known that DTH reactivity plays an important role in allograft rejection. DTH is dependent on antigen-presenting cells (APC) migrating to draining lymph nodes or spleen after endocytosis of antigen, and contacting with antigen-specific virgin T cells. In the past, it was believed that aqueous fluid drainage from the eye entered directly into the venous plexus, which made corneal antigen enter to spleen to induce anterior chamber-associated immune deviation (ACAID). However, recent studies showed that about 20% -30% aqueous fluid went though a minor pathway named uveoscleral drainage, which would allow passage of APC directly to the draining lymph nodes to induce DTH[6]. The existence and importance of such a minor route is supported because it has been shown that antigen-specific T cells accumulate primarily in regional draining lymph nodes and not in the spleen as expected, after injection of the antigen into the posterior chamber of the eye [7]. Recent studies by Yamagami and Dana [3] have shown that removal of cervical lymph nodes delays the rejection of mouse corneal allografts indefinitely, indicating that the draining lymph nodes reside in the cervical region and they are necessary for priming of the immune response to alloantigen. We have confirmed these results and further show that cervical lymphadenectomy can effectively inhibit immune rejection in alkali induced high-risk bed.

Zheng *et al* [8] provided evidence that early lamellar

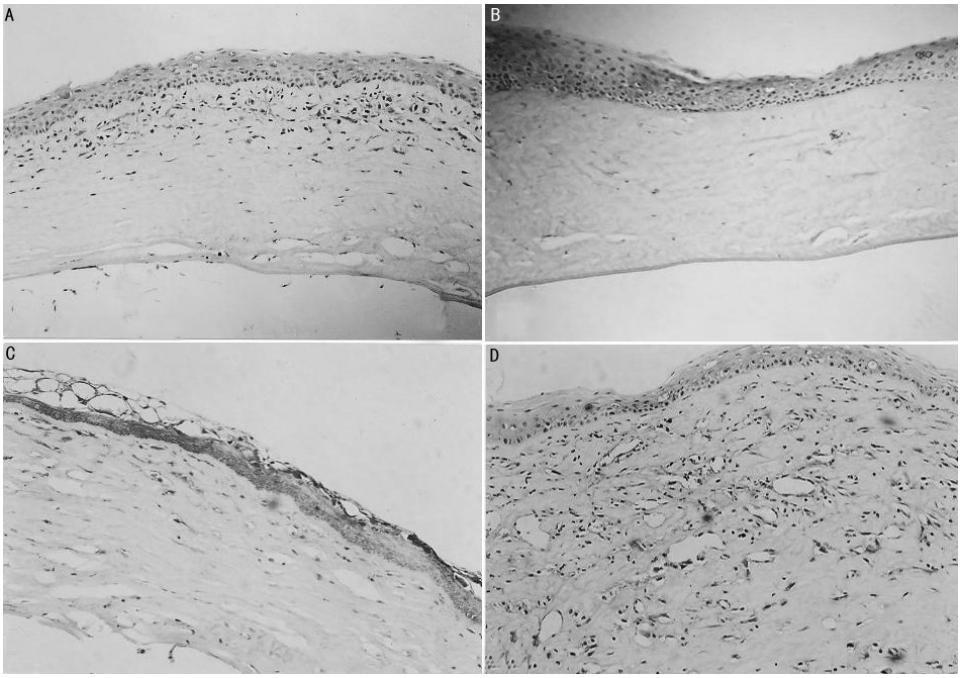

Figure 3 Immunohistochemistry for CD68 in the grafts of each group In group A, there were some CD_{68} positive macrophages localizing in the grafts (A). But the grafts in group B were nearly intact with no CD_{68} immunoreactivity (B). In group C, corneal hypothallus of the grafts was obviously thickened with disorder collagen fibers, and a large number of infiltrating macrophages. In addition, posterior elastic and endothelium layer were nearly disappeared (C). However, there were little infiltrating inflammatory cells in grafts of group D, and the CD_{68} immunoreactivity was weakly positive (D). **Magnification ×200**

keratoplasty after corneal alkali burns could significantly decrease the immune response. The study by Wang *et al*[9] showed conjunctival and corneal transplantation for severely ocular chemical burns performed within 3 weeks was more effective. However, Dua and Blanco [10] argued that it was safe to make transplantation after corneal inflammation resolution. We performed keratoplasty at day 21 after alkaline burns because at that time point, cornea was full of blood vessels but cornea inflammtion had already regressed gradually, which ensure recipient beds to be high-risk but operation to be safe. According to the method of Zhang *et al*[11], we examined the infiltration of macrophages in the graft to show the degree of the inflammation. In addition, to reduce the opportunity of iris injury during the operation, which might cause anterior chamber hyphema, 10g/L pilocarpine was used to dilate pupils before the transplantation.

Our data indicate the crucial role of cervical lymph nodes in alkali burn keratoplasty. However, several questions have not been clarified yet. First, more samples and longer tracking research are needed to observe the complication of cervical lymphadenectomy after corneal transplantation; Second, if lymph nodes can regenerate after lymphadenectomy, the effect of such lymph nodes on allografts should be further studied.

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