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Effect of Amniotic Membrane Transplantation on Limbal Stem Cells in Alkaline Burn Rats

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Abstract

The cornea is located on the outer layer of the eyeball and is very vulnerable to injury. After corneal alkali burn, the epithelial layer of the cornea is seriously damaged due to alkaline saponification. Limbal stem cells, as the regeneration source of corneal epithelial cells, can quickly migrate to the center to cover the wound surface and form new epithelium. This article studies the effect of amniotic membrane transplantation on stem cells of the limbs of alkaline burn rats, which provides strong support for the treatment of corneal diseases. In this paper, ACH was used to encapsulate limbal stem cells to be transplanted to the site of alkali burn of the rat cornea to provide necessary seed cells for the repair of alkali burn of the cornea. To observe its effect on wound repair, it is urgent to provide effective tissue engineering treatment methods for severe corneal alkali burn repair. In this paper, rats were used as the research object. The limbal circular lamelectomy plus central corneal epithelial curettage was used to establish a rat model of complete lack of limbal stem cells in the right eye. The morphology of the ocular surface was observed after 4 weeks. Carrier culture observation, transplantation is performed when the cells are over 90% of the amniotic membrane, the morphology of the right eye of the rat is observed 12 weeks after transplantation, the corneal structure is observed by HE staining, and the differentiation markers of corneal epithelial cells (CEs) are detected by immunofluorescence The expression of CK3+12. After alkali burn of the cornea of rats, the anterior segment of the eye was observed under a microscope, and the corneal turbidity was scored every day. On the 4th, 8th and 12th days after alkali burning, the stained area of the cornea was measured by methylene blue staining to calculate the rate of corneal epithelial healing. The results of the study showed that the corneal repair of rats with alkali burns after 1-3 amniotic membrane transplantation reached a significant standard. The corneal defect area was repaired less than 30%, the matrix infiltration and KP did not decrease and gradually worsened, and a penetrating keratoplasty was given in time.

Keywords: Amniotic Membrane Transplantation, Corneal Alkali Burn, Limbal Stem Cells, Treatment Plan

1. Introduction

At present, in clinical practice, corneal or amniotic membrane transplantation is mainly used to treat corneal alkali burns. However, the former faces two main problems: lack of donors and rejection of the immune system. Amniotic membrane has a composition similar to the horn and conjunctiva (type IV collagen fibers). After burns, collagenase accumulated in damaged tissues is easily degraded, leading to the formation and autolysis of corneal ulcers. Therefore, the treatment of corneal alkali burns should start from two aspects: one is to provide seed cells to repair the corneal epithelium in a timely manner; the other is to provide seed cells for the repair of corneal epithelium. The formation of scar tissue. At present, there is an urgent need to establish a safe and effective alkali burn repair corneal cell transplantation vector to rebuild damaged cells and provide strong corneal disease support.

Amniotic membrane transplantation is an important method for the treatment of alkali burned cornea. Many foreign scholars have conducted research on it. For example, Utheim evaluated the effectiveness of multi-layer amniotic membrane transplantation to reconstruct corneal

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epithelium and stroma in the case of deep corneal ulcers. Design a series of prospective, non-comparative, and interventional cases. Intervention with multilayer amniotic membrane transplantation and cryopreserved amniotic membrane. The main outcome indicators were the integrity of the corneal epithelium and stroma, turbidity and the appearance of the transplanted membrane within 12 months of follow-up [1]. Baradaran-Rafii uses amniotic membrane transplantation to treat ulcers. Separate amniotic membrane is transplanted to fill the matrix layer (amniotic membrane filler), basement membrane (amniotic membrane graft) and wound covering (amniotic membrane patch). All cases received artificial tears, autologous serum eye drops, antibiotic eye drops, topical corticosteroids and sodium hyaluronate eye drops after surgery [2]. Holan, V is to evaluate the effect of amniotic membrane transplantation in the treatment of infectious corneal ulcers, where inflammation is the cause of corneal damage. A prospective study was conducted with 21 consecutive eyes (21 patients). Before permanent or temporary amniotic membrane transplantation, or before combining the two in a small number of patients, adequate antibacterial, antifungal or antiviral agents should be used to eradicate pathogens. Kureshi, a divided the resection surgery into 3 groups, each with 10 rabbit eyes. The first group used a sponge soaked with mitomycin C, and the second group had amniotic membrane transplanted around the scleral flap. The third group is the control group. Calculate the arithmetic mean of the number of fibroblasts and macrophages per square centimeter [4].

In the domestic research on amniotic membrane transplantation to treat eye diseases, Huang, M studied seven eyes of six patients with severe chemical burns (n=5) and thermal burns (n=2). Intervention: remove scar tissue to treat the eye, then place amniotic membrane on the sclera. Simultaneous limbal transplantation was performed from the contralateral eye (n=4) or the donor eye stored at -80 degrees Celsius (n=2). MAIN OUTCOME MEASURES: Measure the reconstruction of the ocular surface epithelium and visual acuity [5]. Zhi,G cut the amniotic membrane into 3x3cm fragments, rinsed in phosphate buffer, and stored in -80 degrees Celsius dimethyl sulfoxide solution until use. In the clinical series, 20 eyes of 20 patients with persistent corneal epithelial defects underwent ocular surface amniotic membrane repair, or as a preventive measure after limbal corneal transplantation [6].

In this article, ACH-damaged encapsulated stem cells are transplanted to the site of alkaline rabbit corneal burn to provide seed cells needed to repair the corneal alkaline burn and observe its effect on performance. There is an urgent need to provide effective tissue engineering treatments for severe corneal alkali burn repair.

2. Research on the Effect of Amniotic Membrane Transplantation on Limbal Stem Cells in Alkaline Burn Rats

2.1. Reconstruction of the Corneal Surface

(1) Persistent corneal epithelial defect and corneal ulcer

In the treatment of corneal ulcers in ophthalmology, although there are many treatment methods and surgical methods, this is still a challenge. The integrity of the corneal epithelium is a very important factor in maintaining the stability of the corneal surface. Mild corneal epithelial lesions can usually be cured without complications. Persistent corneal epithelial defect means that the degree of limb stem cell insufficiency and persistent inflammation is different.

(2) Acute chemical injury

In the early stages of acute chemical injury, prolonged inflammation can prevent epithelial formation, accelerate ulcer formation and dissolution, and even perforate the eyeball. At the same time, eyelid adhesions, eyelid scars, tear film instability and inflammatory granuloma may occur in the chronic phase. Amniotic membrane can effectively reduce inflammation and prevent complications. The amniotic membrane will provide growth factors, protease inhibitors and other biological components to promote the reconstruction of the ocular surface structure.

(3) Application of amniotic membrane in stem cell tissue engineering

The surface of the cornea is very important for vision. The function of the corneal epithelium is essential for maintaining vision. Corneal epithelial stem cells exist at the tip, which is the bridge between the conjunctiva and the cornea. Various ocular surface damage can lead to stem cell damage. The characteristics of stem cell damage include the formation of plaques on the surface of the cornea, photophobia and decreased vision. Only by restoring the number of stem cells in the limbs can the ocular surface structure be successfully created for a long time [7-8].

2.2. Primary Culture of Sheep Limbal Stem Cells and Corneal Epithelial Cells

Stem cells are obtained from the healthy eyes of healthy New Zealand white sheep (approximately 1 year old). The sheep was killed by air inoculation of the surrounding ear veins, the entire eyes were removed, washed several times with PBS solution, and soaked in 400 U/mL double antibody for half an hour.

Primary sheep cell culture: Rinse the anterior chamber with PBS under sterile conditions. With the help of a microscope, take a one-centimeter round tissue from the gray-white junction of the conjunctiva and cornea, cut them into tissue pieces of equal size, with the outer layer facing up, and place them in a 6-cm petri dish , Drip a drop of serum into it (7 small masts per level) or a climbing piece of a 24-well plate (1 tissue block/climbing piece). Add appropriate amount of serum to each tissue block, grow stably in a constant temperature incubator for 4 hours, culture in DMEM/F12 medium containing 15% FBS and 100U/mL diabody for 2 days, and then wait for the cells to move and adhere to stably After that, fill the medium to the normal standard, change the medium every two days, take pictures, and record cell migration and adhesion. The corneal layer was separated under a dissecting microscope to obtain the upper epithelial layer, which was cut into small pieces of 4mm×4mm, and then the epithelial surface was placed in a petri dish containing culture medium. The other steps are the same as above.

After the cells were removed from two adjacent tissues, they were washed twice with PBS. After a few minutes of trypsinization, trypsinization was terminated with serum-containing medium, and cell culture and cell identification were performed.

2.3.Limbal Stem Cells Affect the Repair of Corneal Epithelial Damage

This article uses microsurgery to remove the epithelial tissue of the limbs, and observe the process of repairing corneal epithelial damage in various age groups without limb stem cells. The results showed that after the first removal of the central corneal epithelium, the corneal epithelium of the juvenile group and the control group healed for an average of 8 days, and the corneal epithelium of the adult experimental group and the control group healed for an average of 8 days. The average cure is 13 days [9-10]. There was no persistent corneal ulcer in both groups. The above are derived from the analysis of the SNK method of the epithelial corneal healing rate in each age group.

(1) Research on the ability of different ages to repair corneal epithelial damage

With the increase of age, the ability of the adult group to repair corneal epithelial damage is significantly weaker than that of the young group, and the difference between the old group and the adult group is not obvious. Interestingly, the controls for adults and the elderly are also different. The degree of neovascularization further indicates that the aging of limb stem cells will reduce the function of limb stem cells and is insufficient to resist external damage. Two weeks after the injury, neovascularization began to appear around the cornea of the eyes in each group of experiments. However, in the adult and elderly groups, there were only a few cases of neovascularization, while in the adolescent group there were none. In each set of experiments, there were several cases of invasion from the outside to the inside, which showed that after the removal of stem cells, there is still a large cell reserve without corneal epithelial cell debris.

(2) Research on the role of transiently expanding cells in damage repair

In order to study the cell reserve and distinguish the role of stem cells and transiently expanded cells in repairing damage, this paper performed a second central corneal epithelial injury with a diameter of 9mm. In each group of experimental corneal neovascularization, concentric conjunctival hyperplasia and corneal persistent ulcers were significantly related to aging, most of the controls in each group showed no complications.

After the second injury, the cells in the experiment were further depleted, the central corneal epithelium was missing, and the stem cells of the limbs were removed and could not be replaced, resulting in persistent corneal ulcers. This phenomenon occurs in adults and elderly people. This situation is very obvious in the elderly group, but unlike the expectations in this article, the difference between the adult group and the elderly group is not obvious [11].

In this article, PCNA-labeled cells in the corneal epithelium are mainly rapidly proliferating cells. The results of positive proliferation of labeled cells showed that there was a significant difference between the young group and the adult group ($P<0.05$), but there was no statistically significant difference between the adult group and the old group (P>0, 05). In the absence of limb stem cells, positive cells are transiently expanded cells. With age, the reserve of stem cell function decreases significantly, which may be manifested as a

decrease in the ability to differentiate into transiently expanded cells, leading to the appearance of persistent corneal epithelial ulcers. However, after entering old age, the reserve and function of stem cells may be weakened, but the difference is not obvious in the adult group.

2.4. Culture of Rat Mesenchymal Stem Cells on Amniotic Membrane

(1) Recovery of bone marrow mesenchymal stem cells

The recovery steps are as follows: regularly disinfect the cell laboratory, irradiate with ultraviolet light for 20 minutes, and then ventilate for 10 minutes. Preheat the BMSC rat basic medium and fetal bovine serum in a constant temperature water bath at 37°C for 20 minutes, return 0.25% trypsin-0.4% EDTA and PBS to room temperature and discard; add 5ml solution 1: work in a clean Add penicillin streptomycin, 50ml fetal bovine serum and 5ml glutamine to the 40ml BMSC rat basin on the stage. Prepared as a complete OriCel rat BMSC containing 10% fetal bovine serum for use. Take out 2 special tubes for cryopreservation of cells from dry ice, immediately put them in a 38-degree water bath, and shake them quickly until they are completely melted [12]. Transfer the liquid in each cryopreservation tube to two 15ml centrifuge tubes containing 9ml complete medium; then add 1ml medium to each of these two tubes, and then gently pipette and centrifuge (10 minutes) Then transfer to the above centrifuge tube for 5 minutes. Try to skim off the supernatant, suspend the pelleted cells in 6ml complete medium, measure under a microscope, adjust to a concentration of 2x104/ml, and inoculate them in 3 petri dishes, in a 5% CO2 incubator Incubate at 37ºC and record the date.

(2) BMSC rat communication

The process of replacing the culture medium is as follows: routine disinfection in the laboratory the next morning after recovery, 20 minutes of ultraviolet radiation, and 10 minutes of ventilation. Remove the cells from the incubator, and observe the morphology of the cells in the three petri dishes under an optical microscope. The attachment state, form and attachment state are all good, and the medium can be changed. Take out the complete rat bone marrow mesenchymal stem cell culture medium in the refrigerator at 4°C and preheat it in a hot water bath at 37°C for 20 minutes. Take PBS and place it on a very clean table. Bring it to room temperature. Move the 3 petri dishes to the ultra-clean workbench, aspirate and discard the medium, add PBS to wash once, and then discard the PBS. Slowly add 10 ml of medium to the petri dish. The state of cell adhesion on the three plates was observed again with an optical microscope. No cells float and stick to the wall of the dish. Continue to grow in a 5% CO2 CO2 incubator.

(3) Digestion, transportation and cryopreservation of BMSC rats

The digestion and transportation steps are as follows: When the BMSCs of P2 rats are higher than 80-9%, they are routinely disinfected in the laboratory, irradiated with ultraviolet light for 20 minutes, and then ventilated for 10 minutes. Take out the rat bone marrow mesenchyme from the refrigerator below 4 degrees Celsius, preheat the whole stem cell culture medium to 37°C in a hot water bath, take PBS, 0.25% trypsin-0.4% EDTA and place it on an ultra-clean plate Return to room temperature for use. First move the petri dish to the ultra-clean workbench, then immerse 2ml of 0.25% trypsin-0.4% EDTA in the petri dish, place it in the incubator for digestion for about 1 minute, immediately take it out and discard it, place it under a microscope and observe continuously. When the cells become round and the gap is large, add 4ml of medium to stop the effect. Measure the number of cells in the measuring plate, subtract 1ml and dilute the medium to 1.75x104/ml and accurately seed it on a 96-well plate to determine the cell growth curve. Then take 1ml and dilute it to 2.5x105/ml, and quickly inoculate it on the plate. On the 24-well plate of the upper amniotic membrane, observe and take pictures under an optical microscope to record the growth of BMSCs for 1-8 days.

2.5. Study on Corneal Turbidity Caused by Alkali Burn in Rats

(1) Preparation of vasoactive peptide eye drops

Weigh the closest 2 mg of vasoactive intestinal peptide solid powder with an electronic balance, dissolve it in 2 mL of saline and shake well to dissolve the powder in the solution evenly, and prepare a solution with a concentration of 1 g. Take out 1 ml of the ready-to-use solution and add 1 ml of saline to make the vasoactive intestinal peptide eye drops with a concentration of 0.5 g/L, and add 3 ml of saline to the remaining 1 ml of the ready-to-use solution to make the concentration 0. 25g/L vasoactive intestinal peptide eye drops. Store the prepared reagents in a refrigerator at -20°C. The biological properties of vasoactive intestinal peptide eye drops can remain stable for 3 months in a storage medium at -20°C.

(2) Preparation and administration method of alkaline corneal burn model

Group A was untreated, and groups B, C and D became models of moderate alkaline burn sac of the right eye. Three days before the introduction of the alkaline burn model, 3g/L levofloxacin hydrochloride was used 4 times a day to prevent infection. Wistar rats were anesthetized by intraperitoneal injection of 10g/L hydrated chlorine (3ml/kg) and fixed in the experimental table. 4g/L oxybucin hydrochloride eye drops are used for local anesthesia, and the excess water is wiped off with a sterile cotton swab. Use a microwave oven to pour 10 μL of 1mol/L NaOH solution into a sterile single filter paper round paper with a diameter of 3 m, so that the filter paper is completely saturated, and then the filter paper is firmly fixed in the center. The right cornea ofthe rat was used for 30 seconds and then the conjunctival sac was washed with 10 mL of saline and washed with NaOH solution. Now, in the center of the cornea, the round, gray-white burned area of the cornea can be seen. After the burn, 3g/L levofloxacin eye drops were injected into the eyes 3 times a day. Apply tobramycin ointment to the eyes once a day to prevent corneal infections. Groups B, C and D were all administered on the first day after caustic soda and received 0.9% saline eye drops, 0.25g/L vasoactive intestinal peptide eye drops and 0.5g/L vasoactive intestinal peptide eye drops every day 5 times.

(3) Corneal turbidity

24 hours after the establishment of the alkaline combustion model and before the first drop of medicine every day, the general condition of the front of the eye was observed and recorded under a microscope. The corneal opacity score is based on the Holand standard: 0 points: no corneal opacity, 2 points: mild corneal opacity; 4 points: heavy corneal opacity, visible iris texture. 6 points: The opacity of the cornea is further deteriorated, the iris is invisible, but the pupil is visible. 8 points: The cornea is completely cloudy and the anterior chamber is not visible.

2.6. Establishment of Rat Corneal Transplant Rejection Model

The experiment established 24 kinds of corneal transplant rejection models and eliminated 5 cases of complications. According to Larkin's scoring method, the time rule of the occurrence and development of rejection in this model is studied. On the third to fourth days after the operation, the corneal implants began to appear cloudy and swollen. On days 5 to 7, new blood vessel formation occurred in the implant. In 6 to 9 days, neovascularization invades the implant from the junction of the implant bed and is sutured. New blood vessels are formed most densely, most of which invade the central area of the cornea. All corneal transplants in this group were discarded, and the average survival time was 9.67±1.37 days. After 3 weeks of observation in the model group, the swelling of some corneal implants decreased and the implants began to scar.

List 5 complications: 2 anterior chamber bleeding (occurring during surgery), 1 secondary cataract (occurring on the first day after surgery), and 1 iris prolapse (with sutures on the second day after surgery)) Shedding), 1 anterior iris adhesion (squeezing the anterior chamber on the first postoperative day).

2.7. Establishment of a Corneal Transplant Rejection Treatment Model

This article established and observed the intervention of disease models under local and systemic administration modes. According to clinical observations, there was no significant difference in survival time of corneal implants in the blind control group compared with PBS treatment for each route (P>0.05). Among them, the dosage of the tail vein treatment group was 1ml; the dosage of the eye spot treatment group was 0.05ml. Due to the surface tension of the liquid, a large amount of liquid is eliminated during the simulated flash evaporation process. The subconjunctival injection model should be operated under a microscope under general anesthesia to ensure that the syringe needle is completely buried under the conjunctiva. By injecting fluid, the conjunctival sac can be observed to swell into a crescent shape. The injection volume is 0.1 ml.

2.8. The Impact of Corneal Transplant Rejection

Compared with other solid organ transplants, due to the immune forgiveness mechanism, the chance of immune rejection after corneal transplantation is lower. There are many factors involved in maintaining a state of immune forgiveness, and these factors are interrelated and mutually exclusive. The destruction of the corneal immune mechanism is mainly manifested in the following aspects. New blood vessels invade the corneal graft, Langerhans cells are activated and proliferate, Th1 cytokines are highly expressed, the expression of major tissue antigens are up-regulated, and the chemotactic expression of

inflammatory cells is expressed. The rejection of corneal transplantation is a complex process involving many mechanisms. After tissue organ transplantation, human CD4+ T cells are stimulated by antigens and are divided into two subgroups, Th1 cells and Th2 cells, according to the different cytokines secreted. Th1 cells secrete interleukin-2 (IL-2), interferon-γ (interferon-γ, IFN-γ) and other cytokines that cause delayed-type hypersensitivity; Th2 cells secrete interleukin-4 (interleukin- 4) It has been shown that the immune response mainly induced by CD4+ T cells is the main reason for the failure of high-risk corneal transplantation.

3. Experimental Study on the Effect of Amniotic Membrane Transplantation on Limbal Stem Cells in Alkaline Burn Rats

3.1. Amniotic Membrane Extraction

The placenta was obtained under sterile caesarean section, the placental membrane was removed, the shallow blood stain was washed with sterile saline, washed with 4×106 U/L-1 gentamicin, and then the amniotic membrane was dissected. Flat nitrocellulose filter paper, cut the filter paper with amniotic membrane into 3cm×4cm size. Put the treated amniotic membrane into pure glycerin for dehydration. After 24 hours, transfer it to another glycerin bottle and seal it, then store it in a refrigerator at 4°C. The shelf life does not exceed 30 days. Flush the glycerin in the amniotic membrane graft before use. Put it in a 0.1% tobramycin infusion for 30 minutes, and then use it after repeated washing with saline.

3.2. Techniques and Procedures

(1) Preoperative preparation: Before the operation, the eyelashes of the rat were cut, the tear ducts of both eyes were cleaned, the conjunctival sac was cleaned, and the skin in the surgically disinfected area was cleaned.

(2) Anesthesia: All rats are anesthetized. In other words, 5ml of 2% lidocaine injection + 5ml of 0.75% bupivacaine injection + 75 hyaluronidase units for peripheral injection. Press the eyeball to make it soft and fix the eyeball.

(3) Fixation method of amniotic membrane transplantation: For patients with persistent corneal epithelial defects, drug-induced keratitis and chemical damage after corneal transplantation, amniotic membrane with a high percentage of glycation and glycation should be used. Spread the surface of the cornea and conjunctiva on a flat surface. 10-0 nylon thread attached the amniotic membrane to about 1.0mm outside the conjunctiva, and then took out a piece of hydrated amniotic membrane tissue, laid it on the surface of the cornea and conjunctiva, and continuously fixed it on the 3mm outer tip, and cut off the excess amniotic membrane tissue. For 8 cases (8 eyes) In patients with corneal ulcers, the superficial necrotic tissue was removed first, and then the ulcer was repaired with amniotic membrane, and then covered by the above method. All operations are performed under a microscope, and the accumulation of blood and fluid under the amniotic membrane is contraindicated.

(4) Wear a repeater after the operation, apply mycin and dexamethasone ointment on the eyes, and compress the eyes.

3.3. Check the Eye Treatment

After intramuscular injection of 0.2ml per kilogram of Lu Mianning II for general anesthesia, the rat's ear vein was injected with 0.2ml disiepa, stabilized in the supine position, disinfected, coated and anesthetized with eye drops on the upper eyelid, at the tip The conjunctiva and conjunctiva at the tip of the limb were removed about 3mm outside, and a 2.5% adjusted microscope sleeve with a diameter of 11mm gentian and 9mm Tryphin was used to delimit the superficial inner and outer corneal boundaries. Note that the depth of the needle knife along the marking circle is limited, and the demarcated central corneal epithelium is removed. The depth is about 100~150μm.

3.4. Observation and Collection of Extraocular Image Data of Corneal Rats After Alkali Burn Treatment

The ACH-2 hydrogel encapsulated the primary cultured LSC limb stem cells in the burned part of the alkaline rabbit cornea. The sutures were removed 3 days after the operation, and experimental data were collected on 4, 8 and 12 days.

Model group: On the first day, the alkaline burn area was obviously cloudy, with microvascular effect, and there were more light yellow secretions on the eyelids. On the 4th to 8th day, the alkaline burn area becomes more and more cloudy, the entire cornea tends to become cloudy, and there are still secretions: on the 28th day, the injured area has obvious blood vessel formation, the cornea is obviously swollen, and the central area On the outside, the entire cornea becomes cloudy and secretions are visible.

Treatment group: On the 4th day, the turbidity area of the alkaline burn area was relatively

significantly decreased, there was secretion and no blood vessel formation. From the 4th to the 8th day, the area of alkaline burns was significantly reduced, the ocular surface tended to be transparent with very low visibility; there was no secretion around the eyelids; on the 12th day, the cornea of the injured area was completely transparent and there was no turbidity. The naked eye is similar to normal cornea.

4. Analysis of the Effect of Amniotic Membrane Transplantation on Limbal Stem Cells in Alkaline Burn Rats

4.1. Corneal Epithelial Defects Before and After Surgery

The 44 rats in this group were observed for 3.4±5.8 months after operation. Corneal fluorescein staining was given, and the corneal epithelial repair was observed under a slit lamp. The repair of corneal epithelium after one amniotic membrane transplantation in 28 cases (28 eyes) reached the healing standard, 7 cases (7 eyes) reached the significant standard for corneal epithelial repair, 4 cases (4 eyes) reached the advanced standard for corneal epithelial repair, and 1 case (1 Eye) Corneal epithelial repair has reached the invalid standard. For cases with significant standards and advanced standards, the treatment effect is consolidated. The experimental results are shown in Table 1.

In order to consolidate the treatment effect, the patients with significant standard and advanced standard were given the second or even the third amniotic membrane transplantation. The corneal epithelial repair reached the healing standard within 23.7±9.8 days after operation. For 2 cases (2 eyes) in which corneal epithelial repair was ineffective, one case (1 eye) had a corneal ulcer defect area of less than 30%, and the stromal infiltration and KP were not reduced and progressively worsened. Penetrating keratoplasty was performed. One case (1 eye) B-ultrasound indicated intraocular infection. After the treatment, the condition was not controlled and the eyeball was removed. The corneal epithelium of most rats was completely repaired after the operation, and the difference was statistically significant before and after the operation ($P<0.05$).

Table 1. **Comparison of corneal epithelial defects before and after surgery**

Group	Defect	Complete
Preoperative	42	0
Postoperative	2	38
χ^2		72.4
P		$\ll$ 0.001

4.2. Immunofluorescence Staining of Limbal Stem Cells in the Injured Area

The epithelial layer of normal corneal tissue expresses K3+12 labeled protein (red fluorescence), and stromal cells express Vimentin (green fluorescence). Thirty days after the operation, the eyes or normal corneal tissues were taken respectively for frozen section immunofluorescence staining. Based on this, the relative proportion of the area of the epithelial layer (including the repair area) in the whole corneal area was calculated, as shown in Figure 1.

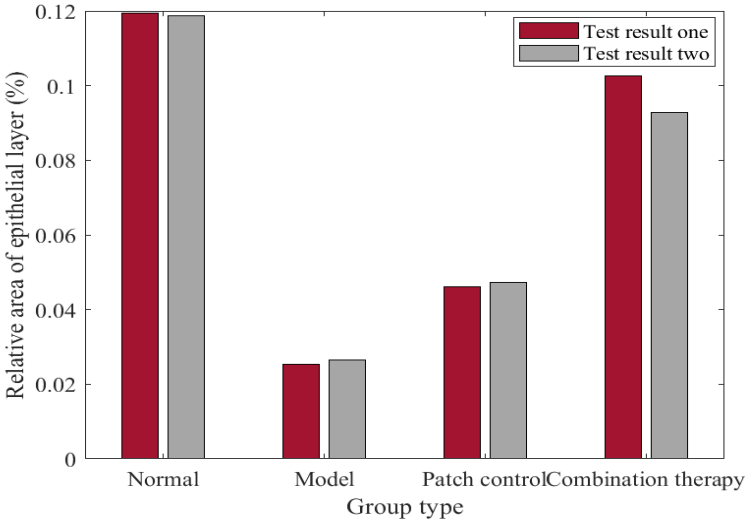

Figure 1. **Comparison of fluorescence staining and epithelial layer in the whole cornea of the alkaline burn area of each group at 30 days after operation**

As shown in Figure 1, it can be seen that the model group cannot form a continuous and smooth epithelial layer only by self-repair; the simple patch control group can also provide a regenerative microenvironment for the proliferation and migration of its own cells, but the effect is limited; while the cell-loaded patch The combined treatment group can not only provide seed cells for the corneal injury area, but also provide a microenvironment for cells to differentiate and proliferate, and promote the complete repair of ocular surface damage.

4.2. In Vivo Observation Experimental Analysis of Corneal Turbidity in Rats with Moderate Alkali Burn

24 hours after the preparation of the alkali burn model, the general condition of the anterior segment of the eye was observed and recorded with a microscope before the first drop of the medicine every day. The score of corneal opacity refers to the standard: 0 points: no corneal turbidity; 2 points: mild corneal turbidity; 4 points : Corneal opacity is heavier, visible iris texture; 6 points: corneal opacity is further aggravated, the iris cannot be seen, but the pupil is visible; 8 points: the cornea is completely turbid, and the anterior chamber is not visible. The experimental results are shown in Figure 2.

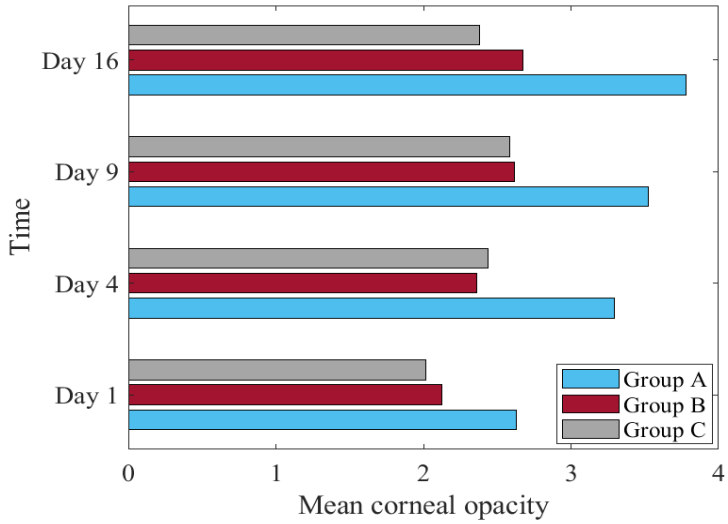

Figure 2. **The average corneal turbidity score of each group at different time points**

As shown in Figure 2, the D group is a normal rat cornea, which is transparent. The first day after corneal alkali burn: the corneal epithelium of the rats in groups A, B and C fell off, the corneal stroma was edema and turbid, and the limbal blood vessels were dilated and congested. Corneal alkali burn on the 4th day: corneal epithelial defects in groups A, B, and C, corneal stromal layer was obviously edema and turbid, and iris texture was faintly visible. There was no statistically significant difference between groups A, B, and C. On the 9th day after alkali burn: The degree of corneal edema in group A was more obvious than that in groups B and C. There was 1 case of retrocornealelastocyst in group A. There was a statistically significant difference in corneal turbidity scores between group A and groups B and C. On the 16th day after alkali burn: In group A, 1 rat had anterior chamber hemorrhage, and 1 rat died after corneal perforation, with diffuse severe corneal edema and unclear iris texture. The degree of corneal edema and turbidity in groups B and C was less than that in group A. The difference between group A and group B and C is statistically significant.

4.3. Comparison of Logmar Vision before and After Surgery

Amniotic membrane transplantation can promote the repair of corneal epithelium through various factors, improve corneal transparency, and ultimately help patients improve their potential vision and improve their quality of life. It was observed that there was no statistically significant difference in uncorrected visual acuity before and after amniotic membrane transplantation, but the postoperative best corrected visual acuity was significantly better than the preoperative best corrected visual acuity, and the difference was statistically significant.

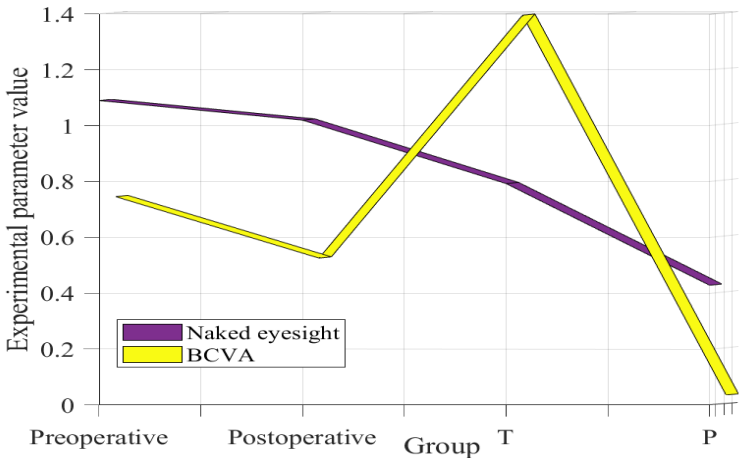

Figure 3. **Comparison of LogMAR vision before and after surgery**

As shown in Figure 3, it can be seen that the corneal repair after 1~3 amniotic membrane transplantation in rats has reached a significant standard. The corneal defect area was repaired less than 30%, the matrix infiltration and KP did not decrease and gradually worsened, and a penetrating keratoplasty was given in time.

4.4. Analysis of the Healing Rate of Limbal Stem Cells after Amniotic Membrane Transplantation

Methylene blue solution can stain the damaged tissues of the cornea. On the 3rd day after corneal alkali burn, the epithelial healing rate of group A is (36.24±3.2)%, compared with group B (41.4±4.28)% and group D (41.87±3.28) % Comparison, the difference is statistically significant (P<0.5). There were statistically significant differences between groups A and B and C on the 8th and 12th day after corneal alkali burn (P<0.5). The experimental results are shown in Figure 4.

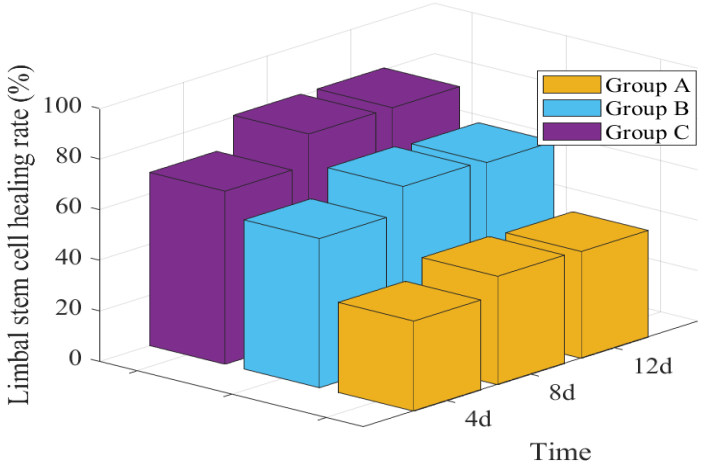

Figure 4. **The average limbal stem cell healing rate of rats in groups B, C, and D at different time points (%)**

As shown in Figure 4, on the 12th day, the staining areas of groups B and C were not enlarged, and the staining situation was relatively stable; the corneal epithelium of group A was stained in patches, and some rats had corneal epithelial defects again. There was no statistically significant difference in corneal epithelial healing rate between group B and group C at each time point (P<0.5).

5. Conclusions

The cornea includes a three-layer structure of epithelial layer, stratum corneum and endothelial layer. Corneal epithelium plays an important role in maintaining corneal vision and clarity. Corneal epithelial cells are mainly formed by the proliferation and differentiation of limb stem cells located in the basal layer of the limb. Studies have shown that LSC grows intensively and gradually

differentiates into corneal epithelial cells. Therefore, LSC plays a central role in the production of corneal epithelial cells. Amniotic membrane transplantation can promote the repair of corneal epithelium and corneal ulcers, reduce corneal inflammation and corneal edema, and restore the patient's vision. Amniotic membrane transplantation is an effective method for the treatment of persistent corneal epithelial defects and refractory corneal ulcers.

Under normal physiological conditions, limb stem cells are not only a barrier between the cornea and conjunctiva, but also play an important role in corneal epithelial regeneration and post-traumatic repair. However, experiments cannot prove that it is the only factor affecting corneal epithelial repair. Whether epithelial stem cells exist only in the limbs needs further research.

The lack of stem cells in rat limbs can lead to loss of corneal epithelial proliferation and reduced limb barrier function, leading to corneal epithelial erosion, increased conjunctival tissue and new blood vessel formation. As we age, stem cells in the limbs are lost and the corneal epithelium is repaired. Physical fitness drops significantly, but it will not change significantly with age.

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