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EXPERIMENTAL STUDY

Transplantation of the allogeneic conjunctiva and conjunctival extracellular matrix

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Abstract: *Objectives:* To investigate the characteristics and effects of allogenic conjunctiva and conjunctival extracellular matrix (ECM) as the substitute of conjunctival tissues.
Background: The symptoms of the frequently-occurring conjunctival injury not only make people ugly, but also lead to blindness, which seriously endanger the quality of life of the patients.
Methods: The bulbar conjunctivas of 6 out of 30 rabbits were prepared into a conjunctival defect model, and the remaining rabbits were randomly divided into 2 groups (n = 12). The conjunctivas of the trial group were repaired by transplanting conjunctival ECM prepared by tissue engineering technology, and the control group received fresh conjunctival allograft. Thereafter, the postoperative conjunctival reconstruction was observed. Their conjunctivas were examined by naked eye, microscope, immunohistochemical and lymphocyte toxicity tests.
Results: Blood vessels of the trial group began to grow into the graft after one week, and the conjunctivas appeared almost normal without immune rejection after 8 weeks. The transplanted conjunctival epitheliums were observed to recover after 4 weeks under light microscope. A large number of invasive inflammatory cells were found in the grafts of the control group 2 weeks after surgery.
Conclusion: Conjunctival ECM is an ideal substitute for conjunctiva, which can be used for the effective surface reconstruction of cornea and conjunctiva *(Fig. 7, Ref. 21)*. Text in PDF *www.elis.sk.*
Key words: extracellular matrix, conjunctiva, transplantation.

The symptoms of the frequently-occurring conjunctival injury include conjunctival cicatrization, symblepharon, eye movement disorders, dry eye, or even pseudopterygium, corneal opacity, progressive corneal conjunctivalization and vascularization, leading to visual loss. The symptoms not only make people ugly, but also lead to blindness, which seriously endanger the quality of life of patients. Therefore, the treatment methods of conjunctival injury are in need of further investigation. The ineffective conservative treatment of large wounds can only be repaired by surgeries. The reconstruction of conjunctival injury is determined by the substitute. Thus, numerous conjunctival substitutes, including autologous conjunctiva, allogeneic conjunctiva and mucosal tissues such as oral mucosa and nasal mucosa, have been widely applied.

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Recently, the transplantation of amniotic membrane has been extensively applied in reconstructing conjunctival injury. With the development of tissue engineering, the use of extracellular matrix (ECM) as the conjunctival substitute has become increasingly popular. In this paper, we compared the performance of conjunctival ECM and allogenic conjunctiva in the repair of conjunctival defects, which demonstrates the potential of conjunctival ECM as the substitute.

Materials and methods

Experimental animals

Thirty New Zealand white rabbits (of either gender) weighing 3 kg without ocular surface diseases were selected. Twelve guinea pigs weighing about 300 g were selected.

Source of acellular conjunctival ECM

The conjunctivas of six rabbits rendered acellular by Beijing Qingyuanweiye Bio-Tissue Engineering Co., Ltd., which were prepared into conjunctival ECM.

Experimental methods

Animal grouping

Twenty-four healthy rabbits weighing 3 kg were randomly divided into 2 groups (n=12). The conjunctivas of the trial group were repaired by transplanting conjunctival ECM, and the control group received fresh conjunctival allograft.

Preparation of animal model

The rabbits were anesthetized by the intramuscular injection of 8 mg/kg phenobarbital and 12.5 mg/kg chlorpromazine. The conjunctival sacs of their right eyes were flushed and administered with 0.5 % tetracaine topical anesthetic eye drops. Their eyelids were opened with an eye speculum. Under sterile conditions, approximately 20×8 mm^2 of the upper right eye conjunctivas of 30 rabbits that were 2.5 mm below corneal limbus were excised. The excised conjunctivas were soaked in normal saline containing gentamicin (80,000 u gentamicin + 100 ml 0.9 % saline) prior to experiment. The trial group and the control group were sutured with conjunctiva ECM and fresh allogeneic conjunctivas by 10-0 nylon sutures. The rabbits underwent dressing changes the next day. The operated eyes were continuously administered with antibiotic eye drops (tid) for 2 weeks. Thereafter, all the sutures were removed.

Visual observation and photographing

Postoperative grafts of the trial group and control group were observed by the naked eye daily, and the external eyes were photographed weekly.

HE staining and observation under light microscope

Tissues of 2 rabbits in the trial group were sampled from the transplantation area 1, 2, 4 and 8 weeks after surgery, those of 2 rabbits in the control group were also sampled 1 week after surgery. After being washed with saline, the tissues were fixed in 10 % neutral buffered formalin solution for 24 h. Thereafter, they were conventionally dehydrated, embedded, sectioned, stained (HE staining) for histological observation. Tissues of the control group were subjected to histological observation and bacterial culture respectively because they dissolved and peeled off two weeks after surgery.

Immunohistochemical examination

The conjunctivas and acellular conjunctival ECM of the trial group before surgery, and the tissues sampled from the transplantation area of the trial group 4 and 8 weeks after surgery, were subjected to immunohistochemical staining.

Lymphocyte toxicity examination

Venous bloods (2 ml) of the two groups were sampled before and 1, 2, 3 weeks after surgery, respectively, and the serum was separated. Then the heparin anticoagulants (3ml) of the donor rabbits were sampled before 30 min of standing. Donor lymphocytes were extracted from the lymphocyte separation medium. Thereafter, the mixture of the donor lymphocyte (20 µl), the receptor rabbit serum (20 µl) and guinea pig serum complement (40 µl) that underwent 1 hour of standing was stained with 20 µl 1 % trypan blue and placed for another half an hour. The survival lymphocytes were then observed and counted under a light microscope.

Statistical analysis

SPSS11.5 was utilized as the statistical software in this study. T test was used. $p < 0.05$ was considered as the statistically significant difference.

Results

Naked-eye observation of transplantation area tissues and light microscope examination of HE staining after surgery

The trial group, postoperative 1st week: there was neovascularization at the graft edge. In the transplantation area, ECM was

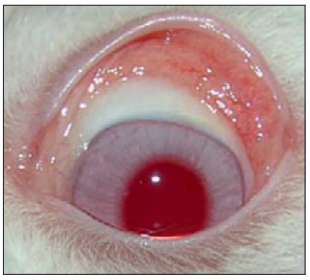

Fig. 1. External eye photograph of the trial group (postoperative 8th week).

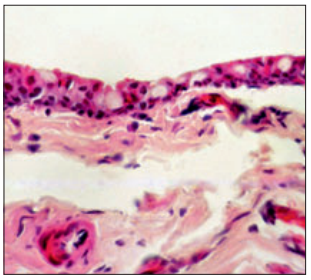

Fig. 2. HE×400 staining of the trial group (postoperative 8th week).

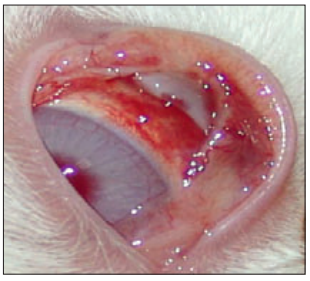

Fig. 3. External eye photograph of the control group (postoperative 2nd week).

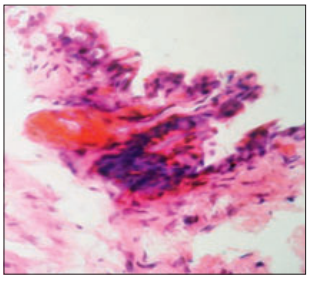

Fig. 4. HE×400 staining of the control group (postoperative 2nd week).

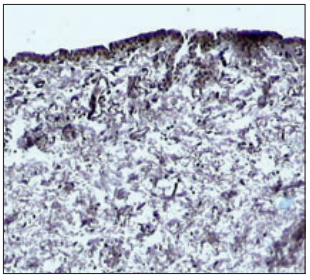

Fig. 5. Immunohistochemistry ×100 of the trial group (postoperative 4th week).

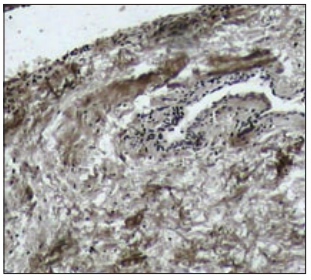

Fig. 6. Immunohistochemistry ×100 of the trial group (postoperative 8th week).

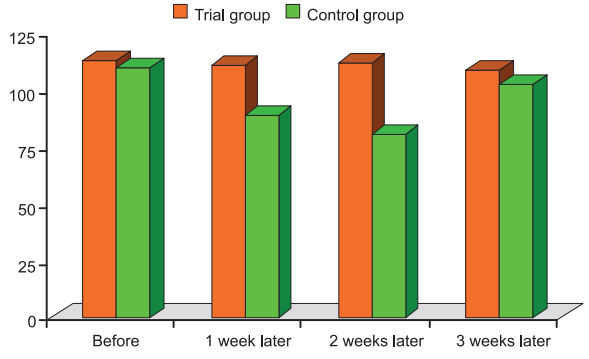

Fig. 7. Lymphocyte toxicity examination.

covered by a single layer of epithelial cells, and there were newly grown blood vessels and a small amount of lymphocytes (under light microscope). Collagen fibers and elastic fibers were found in the matrix. Postoperative 2nd week: a large number of newly grown blood vessels were found at the graft edge. In the transplantation area, ECM was covered by 3–4 layers of epithelial cells that were rich in cytoplasm, and there were newly grown blood vessels and lymphocytes (under light microscope). Collagen fibers and elastic fibers were found in the matrix. Postoperative 4th week: Conjunctival hyperemia was significantly relieved, the vascular textures were clear, and the graft surface was smooth without cicatrice. In the transplantation area, ECM was covered by stratified squamous epithelium low in cytoplasm, and there were newly grown blood vessels and a small amount of lymphocytes (under light microscope). Collagen fibers and elastic fibers were found in the matrix. Postoperative 8th week: the transplantation area appeared almost normal. Under light microscope, the structure of the transplantation area was also normal with 2–3 layers of epithelial cells, goblet cells, blood vessels and collagen fibers (Figs 1 and 2).

The control group, postoperative 1st week: the grafts were gray-white without neovascularization. There were no epithelial cells in the transplantation area, and the infiltration of many inflammatory cells was observed (under light microscope). Postoperative 2nd week: the grafts peeled off, and the conjunctival defects were not repaired. Epithelial cells did not grow into the grafts with tissue necrosis, and there was a small number of negative inflammatory cells (Figs 3 and 4).

Immunohistochemical examination

Four weeks after transplantation, ECM of the trial group that was completely covered by epithelial cells was not stained, and type I collagen in the matrix was positively stained (Fig. 5). Four weeks later, the transplantation area was almost recovered, epithelial cells were not stained, and type I in the matrix was positively stained (Fig. 6).

Lymphocyte toxicity examination

The survival rates of lymphocytes in the trial group 1, 2 and 3 weeks after surgery and before surgery did not differ significantly ($t=0.586, -0.208, 1.206$; $p>0.05$) (Fig. 7). The survival rates of lymphocytes in the control group 1, 2 weeks after surgery and before surgery differed significantly ($t=6.178, 10.986$; $p<0.01$), the survival rates 3 weeks after surgery and those in the normal group differed significantly ($t=2.607$, $p<0.05$).

Discussion

Experimental design

Fornix conjunctiva, which is located between palpebral conjunctiva and bulbar conjunctiva, is the most relaxed part of conjunctiva. This most flexible part is of the thickest tissues and contains rich elastic fibers (1). Besides, fornix conjunctiva contains conjunctival stem cells, thus the corresponding conjunctival defects can be self-repaired. Meanwhile, they cannot be replaced by other tissues. Therefore, they ought to be repaired by transplanting the conjunctiva ECM from the fornix conjunctiva of the same species. Taking into consideration the corneal limbal stem cells, the conjunctivas that were 2.5 mm below corneal limbus were selected. The complete removing of the corneal limbal epithelium may lead to corneal persistent epithelial defects, recurrent epithelial erosions, neovascularization, pseudo-pterygium invasion, or even corneal melting, ulceration and corneal perforation (2). Accordingly, the corneas of the three groups were not affected throughout the experiment. The area less than 5mm^2 of conjunctival defect can restore by self-repair or conservative treatment. Therefore, 20×8 mm^2 of conjunctiva was excised. The tissue repair of conjunctival injury is affected by various factors, which was minimized by simple mechanical injury.

Low antigenicity of conjunctival ECM

The rejection of allogeneic transplantation is mediated by the T cells of receptors targeting the immune response of transplantation antigens. This response is induced by recognizing the allogeneic antigens on the graft cell surface utilizing the T cell receptor (TCR) (3). ECM can be prepared by acellular treatment of conjunctivas that removes the cells containing transplantation antigens. ECM is an extracellular macromolecular non-cellular super composite structure that mainly consists of collagens, proteoglycans and glycoproteins (4). Therefore, the rejection will be avoided after conjunctival ECM transplantation that does not show significant immunogenicity (5). In this study, the growth of new blood vessels into the graft edge 1 week after surgery, a large number of inflammatory cells in the grafts 2 weeks after surgery, and a small amount of inflammatory cells and excreting goblet cells 4 weeks after surgery (the trial group) all resulted from the low antigenicity of ECM. However, conjunctival epithelium that is homologous to corneal epithelium is of significantly high antigenicity (4). In this experiment, the pale grafts and the invasion of a large number of inflammatory cells 1 week after surgery, the peelings off of major grafts, tissue necrosis, the absence of epithelium covering, and the negative bacterial culture two weeks after surgery (the control group) all indicated that the grafts peeled off due to rejection rather than infection. Yang et al (6) and Wu et al (7) have studied allogeneic conjunctival transplantation and found out the rejection reached a maximum in the second week by red blood cell immunity and electron microscopy, respectively. Complement-dependent micro-lymphocytotoxicity examination is classic in the detection of humoral immunity. The statistical analysis of the lymphocytotoxicity examination (3) revealed that there were no significant differences between the trial group before and after surgery, suggesting that there were no significant humoral immune rejections at each time interval (8). In other words, the transplantation of conjunctival ECM into the 12 rabbits of the trial group all succeeded. In contrast, immune responses were all observed in the control group that were transplanted with allogeneic conjunctivas, and major necrotic grafts peeled off in the postoperative 2nd week.

Relationship between conjunctival ECM and tissue injury repair

Wound healing is a complex and highly coordinated process (4), which can be basically divided into local inflammatory response, cell proliferation, differentiation and tissue repair and reconstruction,

involving the interaction between a variety of growth factors and cytokines that co-regulate cell migration, proliferation, differentiation, angiogenesis, matrix deposition and tissue shaping, etc. Wound healing depends on the interaction between cells and ECM (9).

Tissues are all rich in ECM, which functions in connecting cells to maintain the physiological structures and functions of tissues (10). The comparison between the trial group and the control group revealed that the transplantation of conjunctival ECM can accelerate the regeneration of conjunctiva, which is closely related with its structural and physiological functions. ECM mainly comprises (11): collagens that significantly affect cell growth, differentiation, cell adhesion and migration, proteoglycans that affect cell metabolism, growth and differentiation by adhering cells into tissues or organs and participating in gel and sol systems that dominate material exchange and osmotic balance, and adhesive glycoproteins that influence cell adhesion, migration and proliferation.

Conjunctival ECM outcomes

The immunohistochemical examination revealed that the normal and the acellular conjunctival tissues before surgery, and the tissues from the transplantation area of the trial group after transplantation were all type I collagens, indicating the existence of basic collagen structures after acellular treatment. Acellular conjunctiva performs as a biological scaffold that retains the matrix components in the collagen fibers and small blood vessels, which facilitate the ingrowth of receptor cells and new blood vessels. As a result, the newly formed ECM replaces the acellular conjunctiva, leading to the formation of new conjunctival tissues ultimately (12–15). In addition, the components in ECM may change during the repair of tissue injury, such as the decreased type III collagen and the increased type I collagen, which strengthen the repair of tissues (16–21).

Conclusions

In summary, the study herein has demonstrated the following results: 1) conjunctival ECM is of low antigenicity. 2) Conjunctival ECM is able to induce cell growth and accelerate conjunctival regeneration. 3) Conjunctival ECM is a promising substitute for conjunctiva.

With the rapid development of cell biology, molecular biology and biological engineering, one of tissue engineering materials, i.e. ECM, has been extensively applied as the material for repairing defects. Nevertheless, further experiments and clinical trials are still in need in allogeneic transplantation between humans or between animals and humans.

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