Review Article

The development of tissue engineering corneal scaffold: which one the history will choose?

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Abstract: Since the 21st century, the development of corneal tissue engineering technology has been developing rapidly. With the progress of biomaterials, cell culture and tissue engineering technology, tissue engineering cornea has gained great development in both basic scientific research and clinical application. In particular, tissue engineered corneal scaffolds are the core components of tissue engineered corneas. It is the focus of current research on tissue engineering cornea to search for scaffolds with good biocompatibility, high safety and good biomechanical properties. In this paper, the recent research progress of tissue engineering corneal materials is reviewed.

Keywords: Tissue engineering; cornea; scaffold materials

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Introduction

WHO reported on 2010 that 20 millions of patients will become blind because of corneal diseases, which was the second leading cause of blindness in the world (1). Of which 80% patients were able to obtain useful vision through keratoplasty. At present, corneal donors are extremely scarce in China due to the restriction of an aging population, the extensive development of corneal refractive surgery and traditional concepts and other factors. Thus, there are about 5,000 cases per year of allograft keratoplasty in China at present stage (2), far from meeting the needs of clinical treatment. Tissue engineering corneal replacement has become an important means to solve these problems, and is of great social and economic value.

The principle of tissue engineering is that the cells are combined with scaffold carriers in some way, and at the same time, the scaffolds are gradually degraded, and the cells are proliferated, migrated and differentiated on the scaffolds to produce new tissues. The basic element of tissue engineering cornea is the construction technique of seed cells, scaffold materials and tissues. It breaks through the cycle from cell to cell, and reaches higher levels from cell to tissue. Scaffold is a micro environment for seed cell survival, and plays a key role in the construction of corneal tissue engineering and its biological characteristics. Mainly includes the following two aspects: (I) the temporary support, determine the contour to repair the cornea; (II) as a carrier of the adhesion and proliferation of corneal cells, guide the growth or proliferation of cells that are implanted into scaffolds or migrate near the scaffold, providing the basis for cell adhesion, proliferation, migration and differentiation (3). Ideal tissue engineered corneal scaffolds should have excellent optical characteristics, biodegradability, biocompatibility, proper mechanical properties and biomechanical strength. At present, the scaffold materials used in tissue engineering cornea mainly include biomaterials, natural polymer materials and synthetic polymers.

Biological materials

Biomaterial is a kind of natural structure, with many advantages, such as good histocompatibility, rich variety of cytokines and gentle effect on human body. It has been widely used in clinic.

Corneal stroma

Corneal stroma is the key component to maintain corneal transparency. Therefore, it is one of the focuses of tissue engineering research to find corneal stroma substitutes with similar natural corneal stroma structure and biochemical components.

Acellular porcine cornea

At present, the scaffold materials which are the most studied and most closely related to the natural structure are acellular corneal grafts. Acellular corneal stroma is less immunogenic, and its tissue structure is close to that of normal human corneal stroma. It expresses a small amount of antigen epitopes, causing only slight cell rejection (4).

Acellular techniques commonly used include: freezing, oscillation, all kinds of detergent [triton, twelve sodium dodecyl sulfate (SDS), sodium deoxycholate, CHAPS], various enzymes digestion and acid or alkaline treatment, hypotonic or hyperosmotic solution treatment. The above methods can be simply divided into physical method, chemical method and enzyme method. The research focuses on how to remove the cell and antigen components as much as possible while maintaining the corneal stroma, structure, and transparency. Freezing and mechanical oscillations can remove porcine corneal epithelial and endothelial cells, but the components of stromal cells are difficult to completely remove. All kinds of detergents such as triton, twelve SDS, sodium deoxycholate and CHAPS, are also commonly used to prepare acellular porcine corneal matrix method. Du *et al.* (5) found that although triton, sodium deoxycholate and CHAPS can remove the porcine corneal epithelial cells and endothelial cells, there were often residual stromal cell components. The mass fraction of 0.5% twelve SDS will work better on acellular effect, which can remove the cellular components of porcine cornea, almost no cell fragments. However, SDS can reduce corneal epithelial basement membrane glycosaminoglycans (GAG), and damage the basement membrane. Enzyme digestion is also a method of cell removal; common enzymes are trypsin, neutral protease, phospholipase and so on. But the effect is poor when used alone, and it is difficult to completely remove the cells. It is often used in combination with other methods. The combination of methods of physical, chemical, and enzymatic digestion is the most commonly used pattern of acellular cornea treatment to improve the acellular effect. For example, neutral protease II, tris-hydrochloride (tris-HCL), triton, mass fraction of 0.25% trypsin ethylene diamine tetra acetic acid (EDTA), desoxyribonuclease and ribonuclease method can completely remove the corneal cells (6). In addition, the phospholipase A2 can be combined with 0.5% deoxycholate (7), and the human serum is combined with electrophoresis (4 ℃) to remove cells (8). But the latter require fresh human serum, which is expensive. The method of combined cell removal can reduce corneal tissue damage and preserve the transparency of corneal tissue under the condition of removing corneal cells, and is the main research direction at present. Many studies have been done on acellular porcine corneas in China, and some of them have now entered clinical studies (9,10).

Among a number of acellular porcine corneal products, "Ai Xintong"—one kind of cellular corneal stroma, which was independently developed by Chinese scientists, gained approval of the State Food and Drug Administration (FDA) in 2015 and became the world's first tissue engineered corneal product. Zhang *et al.* (11) reported some of the clinical results of this product applied to lamellar keratoplasty for fungal corneal ulcers. A total of 47 cases of patients, the grafts gradually became transparent, 1 month after surgery, the edema disappeared, 6 months after operation, there was no recurrence of infection, rejection and any systemic adverse events. At 3 years after operation, the corneal transparency was significantly higher than 6 months postoperatively. Its current range of application is infective corneal ulcer, clinically ineffective and has not been perforated and temporary coverage of corneal perforation.

Rabbit corneal stroma

Liu *et al.* (12) constructed tissue engineered corneas with human amniotic epithelial cells and acellular rabbit corneal stroma, with a thickness of about 1/2 cornea. It can be seen that the human amniotic epithelial (HAE) stratified epithelium was formed in 2 weeks or so. Hemidesmosome can be seen between the epithelial cells and the basement membrane under transmission electron microscope (TEM), and under scanning electron microscope (SEM) there were abundant microvilli in the epithelium of the amniotic

membrane (AM).

Although the immunogenicity and antigenicity of xenogeneic corneas were reduced by acellular processing, it has a good application prospect in the treatment of ocular surface disease. However, the potential transplant rejection reaction failed to completely eliminate, and the tissue structure is not identical to the normal human cornea. Therefore, it is necessary to further explore more optimized preparation scheme.

AM

AM is the innermost layer of placenta, containing epithelial cells, basement membrane and avascular stroma. Its surface is smooth, without vascular, nerve and lymph. It is about 0.02–0.5 mm thick. The basement membrane and stroma of the amnion contain large amounts of collagen, mainly I, III, IV, V, VII type collagen and fibronectin, laminin and other components. It is these components that allow the AM to act as a "transplanted basement membrane" and play a new, healthy, appropriate role of matrix to promote epithelialization. At the same time, AM promotes corneal epithelial cell migration, promotes corneal stromal collagen fiber proliferation, inhibits subconjunctival fibrosis, inhibits neovascularization, and has anti-inflammatory, antibacterial effects (13). It is a common carrier of corneal epithelium in tissue engineering. Rohaina *et al.* (14) reconstructed corneal epithelium with AM as scaffold and limbal stem cells (LSCs) derived from bone marrow mesenchymal stem cells. After transplantation, corneal neovascularization decreased and transparency increased. In addition, Fan *et al.* (15) constructed tissue engineered corneal endothelium with acellular AM as a scaffold and transplanted it into the cornea after removal of the elastic layer. The cornea remained transparent after surgery. It shows that AM is a good scaffold for tissue engineering cornea, and can be used in tissue engineering, corneal limbus, corneal epithelium and corneal endothelium.

AM has extensive sources, economical price, convenient treatment and preservation, and has effects of anti-inflammatory, inhibiting neovascularization. In addition, AM maintains the viability and function of corneal LSCs. But there are still shortcomings: AM is a biological material, with the possibility of transmitting infectious diseases; in addition, as a carrier, it can be left in the cell layer and the host matrix transplant, resulting in cultured cells loosely attached to the substrate. At present, the basement membrane of AM is more commonly used, and its transparency is better than that of fresh AM. Due to the restriction of the thickness and biomechanical strength of AM, it is difficult to be used in the construction of tissue engineering full-thickness cornea, which is mainly used as a scaffold material for component corneal tissue.

Collagen

Collagen is a kind of structural protein, which accounts for 71% of the dry weight of normal corneal stroma. It is an ideal material for tissue engineered membrane scaffolds. According to different sources, tissue engineering corneal scaffolds can be divided into animal derived collagen scaffold, recombinant human collagen scaffold. Collagen, as an important scaffold material for constructing tissue engineering cornea, provides the carrier environment for the survival of cells; on the other hand, as a component of extracellular matrix, induces cell proliferation and differentiation.

Animal derived collagen

By transfection and viral infection, Griffith *et al.* (16) established the human corneal epithelial, stromal and endothelial immortalized cell line, which were grown on collagen chondroitin sulfate scaffold for co culture. A functional corneal equivalent of normal morphology, transparency, tissue structure and physiological function, which is similar to normal human cornea, was constructed. This study laid a foundation for the development of active tissue engineering cornea which can be transplanted. On the basis of Griffith, Chen *et al.* (17) improved the method of collagen scaffold preparation and improved the mechanical properties of scaffolds.

Corneal endothelial, epithelial and stromal cells, from children who died unexpectedly within one month of birth, were inoculated on the surface and inside and outside the scaffolds, and the corneal tissue was reconstructed by the three-dimensional culture technique of tissue engineering. Tissue engineered corneas were constructed from primary human corneal epithelium, stroma and endothelial cells which were cultured of the same donor for the first time *in vitro*. The scaffold has good biocompatibility, and support the proliferation and differentiation of corneal cells *in vitro*. It lays a foundation for corneal transplantation with collagen scaffold as donor (16-18).

The corneal stroma scaffold made from bovine collagen has been successfully applied to pigs and rabbits for heterolamellar corneal transplantation (19), and good

results have also been achieved. At 6 weeks after operation, the intact stratified corneal epithelium was formed on the surface of the scaffold, and the basement membrane was observed between them. Therefore, it was proved that the corneal matrix scaffold constructed by bovine collagen crosslinking can be well integrated into porcine cornea and induce the regeneration of porcine corneal tissue. It was further proved that the refractive index and diffusion permeability of this scaffold were similar to those of normal human corneal stroma. The toxicity test was negative and the light transmittance of the implants was good. The suture and catgut embedding tests showed ideal mechanical strength. It also supports the proliferation, differentiation and regeneration of corneal epithelial cells *in vivo* and *in vitro*, showing good biocompatibility. Li *et al.* (10) used the porous collagen chitosan sodium hyaluronate complex as scaffold material. The scaffold can promote the proliferation of corneal stromal cells.

Recombinant human collagen

The scaffolds were prepared by crosslinking with recombinant human type I and type III collagen, and the scaffold was used as donor, and lamellar keratoplasty was performed on the pig eyes, and the clinical parameters were evaluated within a period of 1 year. There was no inflammatory reaction or immunological rejection in the cornea.

The cornea remained transparent 1 year after surgery (20). Type I and type III collagen scaffolds promote the differentiation of porcine corneal epithelial cells and the regeneration of neurons. Both can promote the regeneration of corneal stromal cells and epithelial basal membrane *in vivo*. And the comparative study of nerve regeneration (21), among porcine allogeneic corneal allograft and recombinant human type I and type III collagen displayed that, after deep lamellar transplantation, recombinant human collagen scaffolds provide an environment suitable for nerve regeneration in the anterior stroma and the subepithelial region of cornea. At the same time, recombinant human collagen scaffold, avoid the immunogenicity and potential infection risk of animal derived collagen scaffold. Its preparation method was simple, with good biocompatibility, better nerve regeneration ability. As donor materials, it has been used in human corneal transplantation. Fagerholm *et al.* (22) prepared tissue engineered corneal scaffold by recombinant cross-linked type III collagen. A phase I clinical trial of 2 years was performed. Lamellar keratoplasty was performed on nine progressive keratoconus and one corneal stromal scar. The mid-peripheral corneal stroma was transparent and the corneal endothelial function was normal. The clinical examination of the related anterior segment was performed regularly. The results showed that there was no rejection, no infection and neovascularization, and good biocompatibility between the corneal graft and the graft bed. At the same time, the study suggests that tissue engineered corneas can promote corneal nerve regeneration faster than conventional allograft keratoplasty (23). It should be pointed out that the collagen material itself is characterized by fast metabolism and degradation, poor transparency and low tensile strength. Therefore, it is necessary to crosslink it by physical or chemical methods to improve mechanical strength and resistance to degradation. However, the cytotoxicity of residual chemical reagents should be paid attention to during chemical cross-linking. The latest development of the genipin agent with low toxicity, good biocompatibility, can be considered as collagen cross-linking.

Fibrin

Fibrin, a blood coagulation factor, and fibrinogen complete the final step of natural coagulation with thrombin. Fibrin glue, as a new type of biodegradable tissue adhesive, has been widely used in the field of surgery. Fibrin gel can be degraded completely *in vivo*, and the dissolution rate can be reduced by adding aprotinin.

In Talbot *et al.*'s (24) study, rabbit limbal epithelial cells were seeded on fibrin gels (Baxter Hyland Immuno Company, Duarte, CA, USA) with a density of 12,000 cells/cm^2, and the results were achieved in de epithelialization rabbit model of LSC lacking. Through the slit lamp observation and immunohistochemical analysis, K3 and K4 showed that the epithelium was well regenerated at 2 weeks, and the cornea returned to normal after 1 month. This method is well expected to treat LSC deficiency. Fibrin gel has obvious blood and tissue compatibility, non-toxic side effects and other adverse effects. As a biodegradable biomaterial, it has been widely used in clinic and is an ideal carrier for tissue engineering. However, strict screening should be carried out to prevent the spread of blood borne diseases.

Silk fibroin

A natural high molecular fibrin derived from silk, with low immunogenicity, controlled degradation rate, and good mechanical properties (25). It has been used to prepare

tissue engineered corneal scaffolds. Some researchers have constructed a three-dimensional corneal matrix scaffold using silk protein, which has good transparency and supports the growth and proliferation of stromal cells in the scaffold.

Liu *et al.* (26) used silk fibroin as scaffold to cultivate human corneal epithelial cells, and found that silk fibroin could support the growth of human corneal epithelium. Madden *et al.* (27) found that silk fibroin could also support the growth of human corneal endothelial cells, which showed that silk fibroin could be used as a scaffold material for tissue engineering corneal epithelium and endothelial reconstruction. In recent years, silk fibroin, chitosan and collagen were synthesized of composite materials for reconstruction of tissue engineering corneal stroma by scholars at home and abroad, the composite material has good transparency, but biomechanical strength needs to be strengthened (28). In all, silk fibroin membrane supports the growth of primary human limbal epithelial cells, stromal cells and endothelial cells (27).

Other materials

Some scholars reported using poly lactic-co-glycolic acid (PLGA), keratin, chitosan, temperature sensitive materials as corneal stent materials. However, they can only be used in the construction of lamellar cornea because of their poor mechanical strength, so they are difficult to be used as scaffolds for the construction of full-thickness corneal tissue engineering. The internal structure of chitosan is relatively compact, corneal stromal cells are difficult to grow inside, and are commonly used as components of synthetic composites.

Polymers

Polyglycolic acid (PGA) and PLGA

PGA and PLGA are commonly used to construct tissue-engineered corneas, PLGA is a copolymer of PGA, and PGA has been approved by the US FDA for clinical use.

Studies have shown that corneal stromal cells were grown in PGA to form a complex substance and transplanted into the lamellar corneal stroma of rabbits. After 8 weeks, the cornea was transparent and the histological structure was close to the normal corneal stroma. However, the invasion of neovascularization in the later stage may be caused by the degradation of PGA and the increase of regional environmental acidity (29). With regard to PGA and PLGA, they have the advantages of controllable degradation process, good remodeling, high mechanical strength and good biocompatibility. However, the lack of cell recognition signals makes it difficult for cells to implant and acidic degradation products can adversely affect cell activity.

Deshpande *et al.* (30) prepared PLGA membrane by electrospinning, and cultured human limbal epithelial cells. After 4–6 weeks, PLGA degraded to low molecular weight compounds *in vitro*. The tissue engineered cornea constructed by gamma ray sterilization still has regenerative capacity and cell migration after 12 months of low temperature drying and preservation, and it was successfully transplanted in the rabbit corneal alkali burn rabbit model.

Poly(N-isopropylacrylamide) (PNIPAM)

PNIPAM supports the ingrowth of cells and the formation of polymer wrapped cell bodies that are free of cytotoxicity. When the temperature is above 32 degrees, it will settle down from the water. PNIPAM-collagen mixture can promote epithelialization and corneal nerve regeneration, and can be used for stromal lamellar corneal transplantation (31).

Polymer organic synthetic materials have wide range of sources, simple manufacturing process and controllable mechanical properties, but the process of metabolism in human body is not very clear.

Composite

Composites are made of two or more natural and/or artificial materials that combine the advantages of both. It does not change cell compatibility and natural material's original characteristics, and, to some extent, can protect corneal epithelial cells (32).

Garzon *et al.* (33) made tissue engineering corneal scaffolds based on fibrin agarose gel. These scaffolds contained human corneal stromal cells and corneal epithelial cells or human umbilical cord Wharton's jelly stem cells (HWJSC). Application of gas-liquid culture method, immunohistochemistry and fluorescence analysis showed the expression of CK3/12, PKG, ZO1 group, CX43, Cry-αA, Cry-αB, Cry-β, and Cry-ζ and extracellular matrix. This differentiation property can be used to construct biomimetic artificial cornea, which provides hope for patients with LSC depletion. Chitosan and polycaprolactone (PCL) were mixed at different ratios in Liu's research (12) , and corneal

endothelial cells were cultured on the mixed membrane. Cell aggregation, tight junction and extracellular matrix were observed at the 7th day.

Composite materials also have good biocompatibility, histology, physical and chemical properties, biodegradability and transparency, and they have unique advantages as tissue engineering corneal scaffolds. However, the mixing and processing methods of materials, the interaction between different materials and the feasibility of clinical application need further study.

Three-dimensional construction

The three-dimensional construction of tissue engineering is also the focus of current research. Two or three kinds of cells were simultaneously implanted in corneal scaffolds. The corneal grafts with similar morphology and function were constructed by *in vitro* stereo culture. They were used as corneal grafts for the treatment of corneal diseases.

Pang *et al.* (34) constructed corneal epithelium and stromal cells with acellular porcine corneal stroma as scaffold, and successfully constructed tissue engineered corneal anterior lamellar. Tissue engineered cornea was constructed with decellularized porcine corneal stroma as scaffold and rabbit corneal cells as seed cells through dynamic culture (35). The tissue engineering corneal equivalent substitutes containing epithelial, endothelial and stromal cells were constructed. The epithelial cells formed a stratified structure and detected a corneal Pete specific marker CK3. Endothelial cells formed a single layer on the inner surface and expressed aquaporin 1. Corneal stromal cells were injected into the decellularized porcine corneas (DPCs), limbal corneal epithelial cells and corneal endothelial cells were seeded onto the anterior and posterior surfaces of the DPCs (36). On the 14 days and 30 days after operation, it was observed that the cells in each layer of cornea grew well and the number of cells increased.

Conclusions

As a donor cornea, some categories of tissue engineering corneal scaffold has been used in clinic and showed good results. In recent years, with the application of lamellar, deep lamellar keratoplasty and corneal endothelial transplantation in ophthalmology, the basic research and clinical application of tissue engineering cornea have been promoted. It is important for the construction and development of tissue engineering cornea.

AM and silk fibroin are still used only for the study of component corneal construction because they are very thin in thickness. Collagen material has its own shortcomings in terms of transparency and mechanical strength. Natural polymer materials have good biocompatibility, but they have defects of mechanical properties and degradation. The degradation products of synthetic polymers tend to accumulate locally and are not conducive to cell growth. Acellular porcine corneal stroma has an advantage in thickness and mechanical strength. If we can break through the limitations of endothelial deficiency, we can apply it to full-thickness keratoplasty, and fundamentally solve the problem of donor shortage of penetrating keratoplasty.

However, there are still some differences in the physiological structure, biomechanics and long-term clinic efficacy between the tissue-engineered cornea and the normal cornea. In the future, what kind of tissue-engineered corneal scaffold will usher in a breakthrough development, and ultimately become a historical choice?

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