·Basic Research·

Implantation of modified poly 2 –hydroxyethy methacrylate –Polymethyl methacrylate Keratopro – stheses in rabbit and monkey corneas

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Abstract

• AIM: To investigate the bio-colonization of poly 2-hydroxyethy methacrylate (PHEMA) sponge with cornea tissue and evaluate the therapeutic effects of modified porous poly 2-hydroxyethy methacrylate-Polymethyl methacrylate (PHEMA-PMMA) Keratoprostheses (KPro) on rabbit and monkey corneas.

• METHODS: The KPro were made using two-stage polymerization combined with mechanical cutting. The experiments were divided into two groups. In the control group, ten normal rabbit eyes received lamellar implantation of PHEMA sponges. The sponges were obtained 2 weeks, 1, 2, 3 and 4 months after operation. The cell proliferation and neovascularization inside the sponges were observed using light and transmission electron microscopy (TEM) and immunohistochemistry. In the experimental group, the porous PHEMA-PMMA KPros were inserted into the lamellar pockets of eight rabbit corneas and two monkey corneas (stage I operation). The healing process was investigated by slit-lamp microscopy. The anterior lamellar cornea tissues were removed 3 months after surgery, exposing the underneath transparent core (stage II operation). The operated eyes were then followed up for 3-6 months.

• RESULTS: No complications were observed in the control group. Under the light microscope, fibroblasts started to grow into the cornea 2 weeks after operation; lots of cells, accompanied with new blood vessels, invaded into the cornea 2-3 months after surgery. Invading cells of sponge, as well as keratocytes, were positive for vimentin. Under the electron microscope, the invading cells looked healthy and were surrounded by extracellular matrix and collagen. In eight rabbit eyes which received KPro implantation, anterior lamellar cornea melting happened in two eyes after the stage II operation. The remaining six corneas retained their central cores during observation after the stage II operation. Two operated monkey eyes were found no complication throughout the whole follow-up.

• CONCLUSION: The PHEMA sponge can obtain a tight fusion with the host cornea. The modified PHEMA-PMMA KPros have obtained relatively stable results after implantation into animal corneas.

• KEYWORDS: PHEMA-PMMA; Keratoprostheses; rabbit; monkey; bio-colonization

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INTRODUCTION

Corneal diseases are the second common cause of blindness all over the world, next to cataract. As a relatively immune privilege tissue, cornea can easily keep alive in allograft corneal transplantation. However, routine keratoplasty is not successful in treating ocular chemical injury, heat injury, Steven-Johnson syndrome, severe dry eye and other ocular surface diseases with neovascularization. Keratoprostheses (KPro) made of transparent materials can be used in keratoplasty to treat those diseases. But the biocompatibility between KPros and cornea tissue was found to be not good. At present, there is no KPros could be safely used in clinic without any complications.

Many materials have been used as skirts for KPro in the published studies, including carboform [1], the mixture of polybutene and polypropylene, polyfluortetraethylene[2], poly 2-hydroxyethy methacrylate (PHEMA)[3,4], scientists focused KPros on porous supports in recent years.

In China, majority of the corneal blindness patients could not be treated mainly during to shortage of cornea donor tissue. Only few patients could be cured with traditional keratoplasty. So KPro surgery will be used widely in future for patients with severe ocular surface diseases resulting in blindness. At present most KPros were not safe enough, they always lead to serious complications due to poor histocompatibility. Comparing with other KPros, Chirila KPro from Australia has better histocompatibility to integrate with surrounding recipient cornea tissues. But the mechanical tensility of KPros sponge margin is lower, which results in difficulty for suturing and complications after surgery. Based on Chirila KPros, our research not only improved its disadvantages but also took advantage of its strong points. Utilizing PHEMA as chief material, we added some polymethyl methacrylate (PMMA) to strengthen the tensile intensity of KPro. In the meantime, we used this modified porous PHEMA-PMMA KPro in rabbit and monkey corneas and observed the primary results.

MATERIALS AND METHODS

Manufacture of KPro The modified porous one-piece PHEMA-PMMA core-and-skirt KPro had been manufactured by the biomedical engineering Department of Jinan University (gifted). The KPro was made up of central optical core and peripheral porous sponge. The central core was polymerized by HEMA and MMA monomer (mass ratio is 10:1); peripheral spongy skirt was polymerized by HEMA monomer in water. The two regions were joined at their interface by means of an interpenentrating polymer network, a strong chemical junction. There was a layer of PHEMA-PMMA membrane which has been polymerized together with the central core in the bottom of spongy skirt. On this skirt membrane there were many laser holes of 200-300μm size. The resulting device was cut to the required size, curvatured with a diamond-tool lathing machine and stored in sterile balanced salt solution. After autoclaving, the finished KPro had a radius of 7.0mm, a thickness of 0.5mm, a whole diameter of 7.5mm with a central transparent diameter of 4.5mm (optic). The pores of peripheral spongy skirt were between 20m and 60m in size, and porosity was about 65% (Figures 1, 2).

Experimental Animals Eighteen New Zeland rabbits and two rhesus monkeys of either gender were used in this study. The experiments were divided into two groups. Group A: Corneal lamellar implantation of PHEMA sponges were done in ten rabbit eyes. Group B: KPro implantation was done into the corneal lamellae in the other eight rabbit eyes and two monkey eyes.

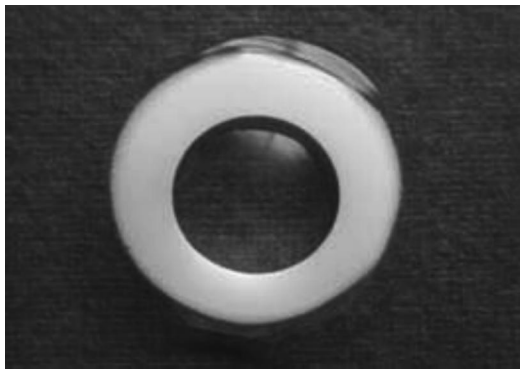

Figure 1 PHEMA sponges

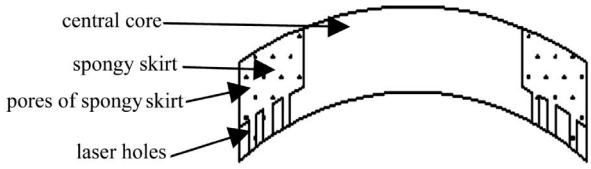

Figure 2 Modified PHEMA –PMMA Keratoprosthese KPro has a radius of 7.0mm, a thickness of 0.5mm, a whole diameter of 7.5mm with a central transparent diameter of 4.5mm

Surgerical Procedure

Implanting PHEMA porous sponges into the lamellar pockets: group A Under general anaesthesia, we dissected the partial anterior cornal (diameter is 9mm), implanted the PHEMA sponges between the cornea lamella, and sutured the anterior cornea with 10-0 nylon. Slit lamp pictures of these eyes were taken on 2, 4 weeks, 2, 3 and 4 months after operation. Two pieces of corneas were taken out at every period for histopathology under light microscopy and transmission electron microscopy (TEM); and immunohisto-chemistry was also done.

Keratoprostheses implantation: group B The KPro was implanted through a two-stage surgery procedure. The interval between stage I and stage II was 3 months.

Stage I: Under general anaesthesia, 9mm trephine was used to imprint an annular mark on the cornea with the papillary as central point. A partial cut was made in the cornea involving peripheral superior 270° according the mark. The depth of cutting was about half of corneal thickness. We dissected the anterior corneal flap from posterior cornea, the dissection continued as a pocket of 9mm diameter within the inferior cornea. The flap was retracted inferiorly, allowing a central trephination of 3mm to be made in the posterior lamella of the cornea. The KPro was then positioned within the pocket so that the center of its optic lay over the posterior opening. The skirt of the KPro was sutured to posterior lamellar cornea with 10-0 nylon. The anterior lamellar cornea was replaced and sutured with posterior lamellar cornea *in situ* with 10-0 nylon. At that time, KPro was held between anterior and posterior lamellar cornea, injecting physiological saline solution into anterior chamber

from side incision of cornea. Sub conjunctival injections of dexamethasone 2.5mg and gentamicin 20mg were given. Tarsorrhaphy was done and kept for 48 hours. Topical Tobradex eyedrop was given four times daily for the first postoperative month, and Tobramycin 3g/L ointment was given every night until the second stage of surgery.

Stage II: The anterior layers over the KPro optic were trephined with a 3mm dermatologic punch to expose the optic as a full-thickness corneal replacement. Then tarsorrhaphy was done and kept for 48 hours. Postoperatively, TobraDex eyedrops 4 times per day and 5g/L tetracycline ointment every night to animals for 4-6 weeks.

Histopathology examination Cornea tissue was fixed in 40g/L paraformaldehyde, and then embedded in paraffin. After the sections were made, haematoxyline-eosine staining was done and observed under light microscope.

Immunohistochemistry examination Streptavidin biotin-peroxidase complex (SABC) technique was carried out. First antibody was mouse anti-rabbit monoclonal antibody (dilution was 1:50), second antibody was goat anti-mouse IgG. All the reagents were come from Wuhan Boster Company.

Transmission electron microscope examination Cornea tissue was fixed in electron microscopy stationary liquid, dehydrated, soaked, blocked, sectioned, stained, and observation under TEM. KPro and neighboring tissue (including cells) were seen with low power lens. Structure of cell organs, extracellular matrix, collagen and angiogenesis were observed with high power lens.

Ultrasonic biomicroscope and B –mode ultrasound examination Three months after the second stage of operation, the eyes of two-monkeys were subjected to Ultrasonic biomicroscope (UBM) and B-mode ultrasound (B-scan) to see the situation of anterior chamber angle, vitreous body and retina.

RESULTS

Group A

Slit lamp examination We didn't detect the complication after sponge intralamellar implantation in 10 rabbit eyes. In early stage after surgery, there was no obviously response in either anterior chamber or pupil. Ten to fourteen days later, there was angiogenesis in upper part of limbus. Till three weeks later, there was much angiogenesis; some of it came near to the edge of PHEMA sponge. Six to eight weeks later, plenty of angiogenesis appeared in corneal stroma around the sponges. Three to four months later, PHEMA sponges remained stable in lamellar pocket and there were no new blood vessels (Figure 3).

Histopathology results Two to four weeks after implantation, cells were seen growing into the margin of PHEMA sponge; most of those cells were fibroblasts, others were inflammatory cells. We also found some inflammatory cells, mostly lymphocytes, in nearby corneal tissue. Two months later, fibroblasts and new blood vessels dispersed in the PHEMA sponge; collagen fibers in nearby cornea arranged orderly with few inflammatory cells infiltrated. Three to four months later, lots of fibroblasts and angiogenesis occurred in sponge without inflammatory cell infiltration (Figure 4A, 4B). Corneal tissue near the sponge showed regularly arranged collagen fibers and few eosinophilic granulocytes.

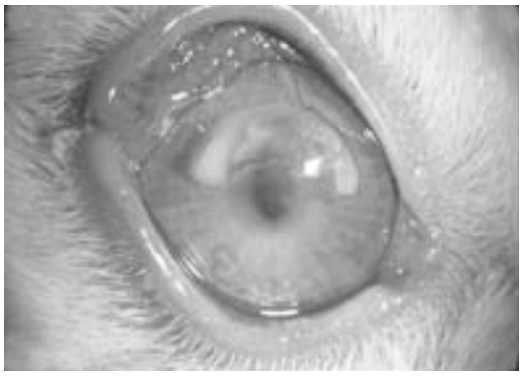

Figure 3 Three months after sponge into intra lamellar cornea, new vessel invasion into sponge pore

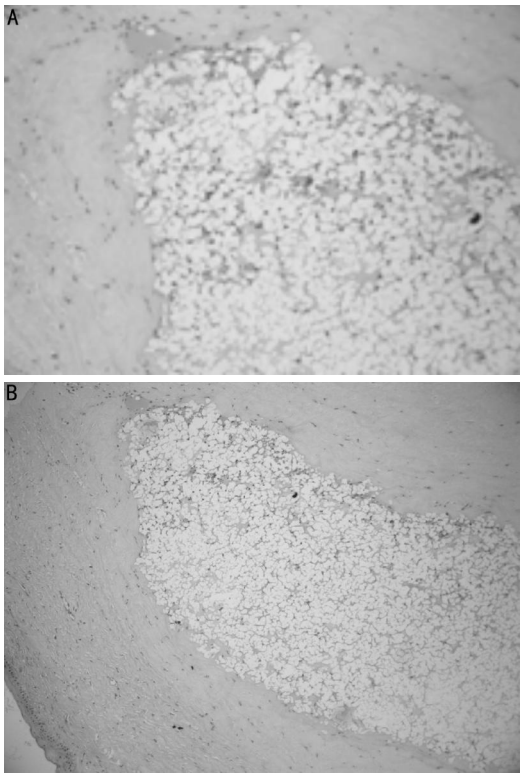

Figure 4 A: Haematoxyline eosine stain, fibroblast and new vessel seen into the pore of the sponge 3 months after sponge into intra lamellar cornea (200×); B: Haematoxyline eosine stain, no obvious inflammatory cells intiltrated into the pore of sponge 3 months after sponge into intra lamellar cornea (200×)

Immunohistochemistry results We got sections of corneal tissue at different periods. In each time, stroma cells of cornea were positive to Vimitin and full of filemot cytoplasm.

The fibroblasts in sponge tissue held between lamellar corneas were positive to Vimitin immunoreactions. Their cytoplasm was filemot and nucleolus was blue (Figure 5).

TEM results Fibroblasts grew into the holes of sponge two to four weeks after surgery. Till two months after operation, most fibroblasts grew up between the pores in the sponge.

Plenty of chondriosomes and rough endoplasmic reticulums were observed. Three to four months later, more cells growing into the pores of sponge. We could see cytoplasmic processes extend along the interspace and some of them superpose each other under low power microscope. We also observed that cell organs, chondriosomes and endoplasmic reticulums were active under high power microscope (Figure 6). There were angiogenesis and vessel endothelial cells in some fields of view.

Group B

KPro in rabbit cornea In eight eyes PHEMA-PMMA KPros was implanted. Two months after the stage I surgery, anterior lamellar cornea gradually dissolved in two of those and hence, further follow up was discontinued. KPro in one eye extruded one month after operation without cornea melting or perforation, so we continued to operate the second part on it.

There five eyes had no obvious complication and received the stage II surgery (Figure 7).

Six eyes received Stage II operation. During the observation, two eyes had little transparent crystal in central core, while the other four eyes were completely transparent. Following three to six months, we didn't find complications such as KPro extrusion, retroprosthetic membrane, anterior synechia, posterior synechia or cataract. Both central and peripheral anterior chamber were normal. However, in four eyes fibrous membrane in front of the KPro, occluding the pupil area was noted.

In the other 2 eyes central cornea was still transparent till end of the research work (Figure 8).

KPro in monkey corneaKPro implantation was done in two monkeys' eyes. There were minor complications in either in stage I surgery or stage II surgery.

In pupil area, there were little exudation in anterior chamber after stage I surgery, and the exudation were absorbed within one week. During one to two weeks after surgery, there was low-grade edema in corneal stroma, and there were hydrops between KPro and posterior lamellar cornea. We examined the eye pressure with finger. The eye pressure was normal or low. The pupil was round; neither anterior synechiae nor posterior synechiae were observed. One month later, there was angiogenesis in anterior lamellar. Two to three months later, ocular surface was stable, so we could prepare to do stage II surgery (Figure 9).

During stage II surgery, we didn't find adhesion between anterior lamellar and the KPro central core, and there was

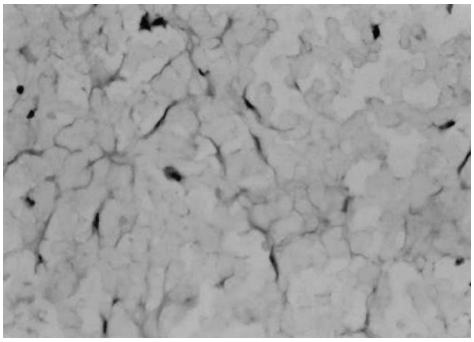

Figure 5 Immunohistochemistry, fibroblast in the sponge is positive for Vimitin (400×)

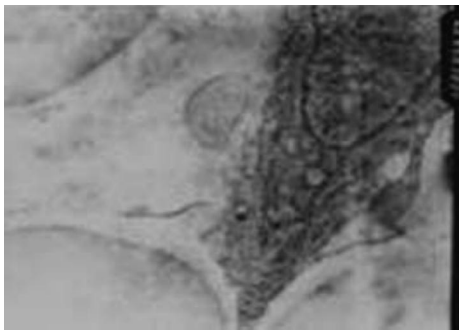

Figure 6 TEM fibroblast in the sponge spore (12 000×)

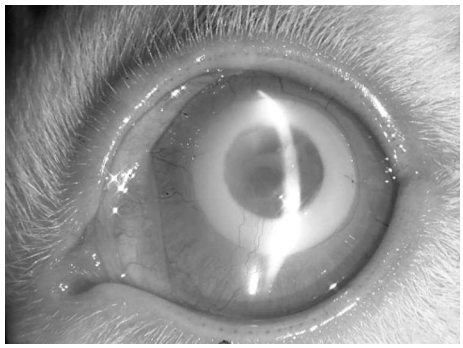

Figure 7 Three months after stage I of KPro

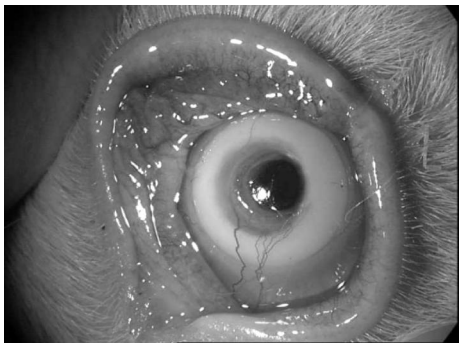

Figure 8 Two and half months after Stage II of KPros implantation in the rabbit eye, the central core is transparent

no leakage, exudation or posterior membrane. The depth of central chamber was normal. The pupil was round and there was no cataract. There was little transparent crystal in the central core of one eye. The other eye was transparent. Three months later, central transparent area remained clear. Neither anterior fibrous membrane nor posterior membrane

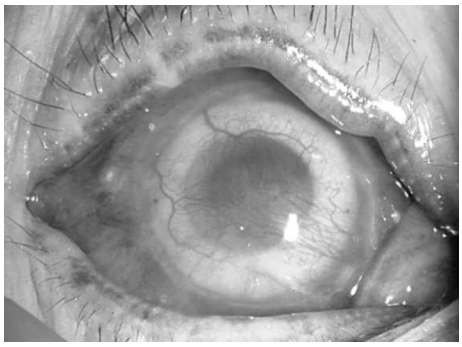

Figure 9 Three months after Stage I of KPros implantation in monkey eye, the anterior cornea is slightly opacity

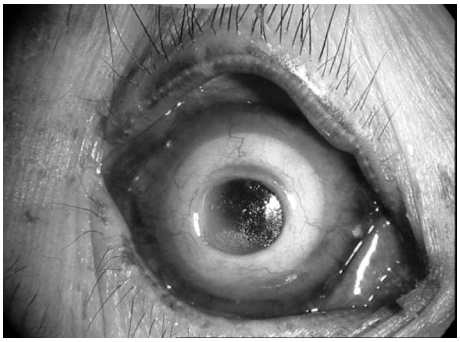

Figure 10 Three months after Stage II of KPros implantation in monkey eye, pupil can be seen without exudation

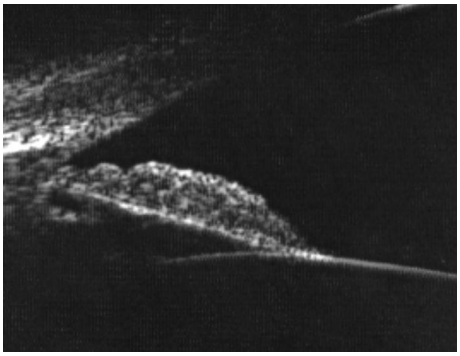

Figure 11 Three months after Stage II of KPros implantation in monkey eye, anterior angle is open seen by UBM

was observed. Anterior chamber and lens were normal (Figure 10).

UBM and B –scan UBM: Anterior chamber angle was open in each quadrant, root of iris was flat and no anterior synechia in 1 eye. There was slight bulge in root of iris, anterior synechiae in 9-10 clock and narrow anterior chamber angle nearby in another eye (Figure 11).

B scan: There was no vitreous opacity or retinal detachment in both eyes.

DISCUSSION

The Research of Porous Skirt Early in 1789, De Quengsy suggested using KPro to replace turbid cornea, but the research about KPros developed slowly since that time. The main complication of prosthokeratoplasty was, and still is, the spontaneous rejection of prosthesis, known as "extrusion". The modern period began in the late 1940s, with the introduction of synthetic polymers as materials for KPros [5]. Scientists gradually recognized that corneal cells needed to grow into porous skirt of KPro in order to make the adequate healing and bio-colonization between the skirt region of KPro and the host cornea. Therefore, researchers concentrated on the porous skirt of KPro. PHEMA was relatively superior to other high molecular materials [6,7]. In this study, we did animal experiments with PHEMA sponge. The histopathology results showed that cells had grown into PHEMA sponge two weeks after operation. Then angiogenesis began to occur along with cells' growth. Because of operative wound and slight foreign body reaction the spongy material in lamellar pocket aroused inflammation in a short time. But inflammation became less as would started healing. Immunohistochemistry results showed that the cells in porous sponge were homologous with corneal stroma cells. Both of them belonged to fibroblasts of cornea stroma. TEM examination showed that cells grew exuberantly in pores of spong. The rough endoplasmic reticulums and chondriosomes were active and there were much collagen and extracellular matrix. It means that PHEMA sponge had good histocompatibility to let cells grow into it. Furthermore, those cells could secrete collagen and extracellular matrix to combine with the corneal tissue authentically.

Modified KPros PHEMA, a hydrophilic polymer, is a biomaterial with a convincing record of ocular tolerance in previous applications (contact lenses, intraocular lenses, intracorneal inlays). PHEMA gel is transparent, stretchy and has good air permeability. The characteristics of PHEMA are just like those of cornea. So it is an ideal material for KPros. The results of our research and other similar studies have proved that PHEMA sponge had good histocompatibility to heal within the corneal tissue [8,9]. Thus it was the best material to be porous sponge of KPro. But no matter the transparent gel or porous hydrophilic sponge, their tenacity was too bad to endure certain pressure. The Australian scientists have studied KPros for many years. They applied many different crosslinking agents to strengthen the mechanical resistance of material. We aimed to find a solution for this problem.

PMMA was a transparent carbonaceous macromolecule organic material. It had been made into intraocular lenses successfully for many years [10,11]. Comparing with PHEMA, PMMA had better tenacity and stretching resistance. But it was hard for PMMA to combine with corneal tissue.

After doing some experiments, we found that when we added some PMMA into PHEMA we would synthesize homogeneous PHEMA-PMMA water gel without affecting

the aggregation of PHEMA. The stretching resistance of this material was at least two times higher than simple PHEMA water gel. There was a thin layer of PHEMA-PMMA gel on the bottom of modified KPro spongy skirt. Because of many laser holes in this layer provided good environment for suturing and tissue healing, thus allowing cells to grow into the interspace of porous sponge skirt so that complete biological healing between KPros and host corneas occurs . Theoretically, the stretching resistance of modified KPro was better than the Australian Chirila KPro. But it should be further verified by animal experiments if KPro could reduce the incidence of KPro extrusion after implantation.

Results of KPro Implantation and Other Unsolved Problems The extrusion of KPro after implantation was the biggest problem for a long time in this field. In this study, we improved the components and patch design of KPro. The modified KPro seldom emerged in our observational duration. That probably related to the improvement of patch design. In addition, the results of long time observation after implantation were still dependent on follow-up survey.

The chief complication of KPro implantation was the formation of anterior proliferative fiber membrane. The incidence increased with time. The membrane completely covered visual area in some cases. The possible reasons for this could be: 1) Patch design was needed to modify further. The chief reason of anterior membrane formation was that central lens was umbilicate. So fibroblasts grew to the front of lens and covered visual area. At present, we are improving this kind of patch and increasing the height of central lens in order to decrease the incidence of anterior membrane formation. 2) Inflammation on ocular surface was involved in anterior membrane formation. Reinforcing the using of local anti-inflammatory agent could improve it. 3) The improvement surgical technique i.e simple central anterior lamellar resection be replaced with lamellar resection combined oral mucous membrane transplantation, the incidence of anterior membrane formation would be decreased.

There was anterior lamellar melting in 25% cases after stage I operation. That maybe correlated to the local ischemia and hypoxia early after KPro implantation. Theoretically, if we used bulbar conjunctiva to cover the KPro in the end of stage I operation, the incidence of cornea melting would be decreased. That is because bulbar conjunctiva could not only strengthen the KPro but also enhance the supply of nutrition. In conclusion, the PHEMA sponge could obtain a tight fusion with the host cornea. KPros were stable 3-6 months after operations. We did not observe many serious complications.

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