• Basic Research •

Effect on rabbits' intraocular structure by cross-linked hyaluronic formations as vitreous substitute

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Abstract

● **AIM:** To develop a new material for retina filling and to investigate its effect on intraocular structure and histocompatibility in rabbit eyes.

● **METHODS:** The polymer-derived hyaluronic acid (HA) was formed by UV light cross-linked with N-vinyl-pyrrolidone. Vitrectomy was performed in the rabbits, and then cross-linked HA hydrogels at different concentrations were injected. Intraocular pressure measurements, cornea check-up, and B-ultrasound examination were performed during the follow-up period. After six weeks' follow-up, the rabbits were sacrificed, and both eyes were removed for hematoxylin-eosin (HE) staining, and the polymer materials were observed under electron microscopy.

● **RESULTS:** The particle size of the cross-linked HA hydrogels was mainly around at 100 nm. After vitrectomy and injection into vitreous cavity optical coherence tomography showed that the polymeric material HA had no significant effect on the overall thickness of the retina. The intraocular pressure returned to the normal level gradually at week 4. B-ultrasound results revealed that there is no significant change in the eye tissue given to HA material. The pathological and transmission electron microscopy results showed no obvious pathological change in the primary cells and rod cells under the retina tissue.

● **CONCLUSION:** HA-based cross-linked biopolymers has good biocompatibility in rabbit eyes, showing a promising potential as vitreous substitutes.

● **KEYWORDS:** rabbit; vitreous; cross-linked hyaluronic acid; retina; biocompatibility

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INTRODUCTION

Clinically, vitrectomy with vitreous tamponades is often the main treatment for retinal detachment. The characteristics of the vitreous tamponade will directly affect the success rate and prognosis of the operation. At present, the commonly used vitreous substitutes in the clinic are SF6, C3F8, and silicone oil[1]. However, most of endotamponades have certain defects in various surgical procedures, such as existing gas and liquid tamponades, which could not provide good support for the lower retina, resulting in a higher failure rate of retinal detachment under the lower hole. Therefore, in order to improve the healing of the underlying retinal tears, there are two heavy silicone oil products approved as intraocular tamponades for clinical use in Europe, Oxane HD (silicone-fluorinated paraffin mixture) and Densiron (silicone-heavy water mixture)[2]. Heavy silicone oil solves the problem of the lower retinal detachment in some extent and help for the retina reattachment, whereas many of its complications have also been observed in clinical applications, such as cataract, intraocular light-to-moderate inflammatory response and increased intraocular pressure, *etc*[3-4]. Therefore, vitreous tamponades with non toxic, of inertia, and hard to biodegrade may the basis for good biocompatibility. At the same time, considering the structural characteristics of the eye, it also requires a certain elasticity, transparency, and refractive index close to glass, good water and oxygen permeability. Finally, fluidity and stability could be further considered, which mean after injection into the vitreous cavity through tiny incision, it would change into a certain solid phase according to body temperature, with which not easy to move. These aspects would be the main consideration or characteristics of an ideal endotamponades[2].

Natural hydrophilic polymers, such as hyaluronic acid (HA) polymers that meet these needs are found by the researchers[5]. HA, an important component of the vitreous body, is widely used in ophthalmic surgery, but its natural form is not suitable as a vitreous substitute because it degrades rapidly and may cause increase of intraocular pressure[6]. Low HA is a glycosaminoglycan copolymer that can be used as a basic material for developing vitreous substitutes. The polymer is formed by D-glucuronic acid and N-acetyl-D-glucosamine through the alternating connection of β-1,4 and β-1,3

glycosidic bonds, with its axial hydrogen atoms forming a non-polar hydrophilic surface, which results in a spiral-like band structure, in physiological solution an expanded random coil structure and forms a viscous compound with a expansion coefficient up to 10 000[7]. Barth *et al*[8] have extensively studied the synthesis, characterization, and application of hydrophilic polymers as vitreous substitutes. The application of hydrophilic polymers has greatly improved biocompatibility, but challenge of long-term stability and small size needle injection unavailable still exist[9-10]. In this study, two different concentration of HA polymer materials obtained by UV cross link were compared in optical and rheologoical properties, and their effect of long-term biostability and histocompatibility in rabbit eyeballs were investigated.

MATERIALS AND METHODS

Ethical Approval The study was approved by the Medical Ethics Committee of Ningbo Eye Hospital and supervised throughout the process. All procedures in this study complied with the ARVO statement for the use of animals in research.

Materials HA from the cockscomb was selected and had a molecular weight of 1-4×10^6 for the synthesis of cross-linked hydrogels. Cross-linked HA was produced by Hangzhou Xiehe Medical Biological Preparation Co., Ltd., with two specifications of 24 mg/mL (No.S29171002) and 40 mg/mL (No.S15171101). The cross-linked sodium hyaluronate gel is prepared by sodium hyaluronate powder (a product of fermentation) undergoing processes such as cross-linking, dialysis, granulation, filling, and sterilization. The appropriate hydrogel was chosen for further evaluation among two different concentrations of cross-linking components through two different cross-linked ways in the previous study.

Research Animals and Grouping Twenty healthy adult New Zealand white rabbits were provided by the Experimental Animal Center of the Medical School of Ningbo University. The rabbits weigh about 1.8-2.2 kg, male, and have no anterior segment lesions in both eyes. The right eyes were selected in the research group while the left eyes were taken as normal control group. After performing vitrectomy, the research eyes of rabbits were divided into three groups: Group A, injection with 24 mg/mL cross-linked sodium hyaluronate gel; Group B, injection with 40 mg/mL cross-linked sodium hyaluronate gel; Group C, injection with steriled 0.9% saline, mean normal control.

Rabbit Vitrectomy Conventional slit lamp microscope, indirect ophthalmoscopy, non-contact tonometer for intraocular pressure measurement, and fundus photography were performed before surgery to exclude eye diseases. All eyes were applied with 0.5% levofloxacin eye drops before surgery. The conjunctival sac is then cleaned and the compound tropicamide is applied for complete mydriasis. The basic steps of the operation are as follows: The rabbits were anesthetized by intraperitoneal injection and oxybuprocaine hydrochloride eye drops (Benoxel) for topical anesthesia. About 3 mm from the limbus, the scleral incision was placed on the supratemporal, supraorbital, and infratemporal area. The cutting head, optical fiber, and perfusion head were placed respectively. The flat concave contact lens was placed on the cornea. Subtotal vitrectomy was performed under a surgical microscope.

The intraocular pressure was 15-25 mm Hg detected before surgery. During surgery, avoid surgical instruments that hurt the lens and retina. After the completion of the subtotal vitrectomy, the gas-liquid exchange is carried out, and the sterile air is continuously pumped by the perfusion head with air pressure of 30-35 mm Hg. The intraocular balance fluid is exchanged from the body. The 1.0-1.2 mL of two different concentrations of cross-linked HA were slowly injected from the perfusion tube into the vitreous cavity in the research groups, 24 mg/mL for research group A and 40 mg/mL for group B. Tobramycin eye drops were applied 3 times/d for 3-5d. Compound tropicamide sputum was applied for mydriasis, 2 times/d for 3d. The intraocular pressure was measured at 1, 2, 3, and 4wk after surgery. Clinical observation was made at different time points after the surgery: left and right corneas were observed at ld, 1, 2, 3wk, 1 and 3mo after the surgery. B-ultrasound and pupil dilation were taken for complications such as lens opacity, vitreous hemorrhage, endophthalmitis, and others.

Pathological Examination The research animals were sacrificed by air embolization 3mo after the surgery, and the eyeballs were removed (24 eyes; 12 eyes in the research group and 12 eyes in the control group). The retinal tissue (10×10×1 mm^3) from the optic disc in the vertical direction of the posterior pole was fixed with formalin, dehydrated with gradient ethanol, embedded with paraffin, and made into ultra-thin sections for hematoxylin-eosin (HE) staining. The steps are as follows. Sampling fixation: the tissue was quickly cut off and placed in the fixation solution. Gradient dehydration: the tissue was soaked in 50%, 70%, 90%, and 100% ethanol for 2 times, 15min each, and in 100% xylene for 2 times, 15min each. Embedding: the tissue was soaked in 56 degrees paraffin wax for 4 times, 30min each, was put into the embedding box and embed with 56 degrees paraffin wax and sectioned into 5 μm slices, which were stained with HE staining and observed under an optical microscope.

Transmission Electron Microscopy Transmission electron microscopy is performed to observe the ultrastructe changes. Sample preparation: the tissue was rapidly cut into 1×1 mm^2 size, fixed with 2.5% glutaraldehyde (containing 0.1 mol/L phosphate buffer) for more than 2h, rinsed with 0.1 mol/L phosphoric acid solution for 15min/3 times, fixed with 1%

osmic acid for 2-3h, followed by gradient dehydration: 50%, 70%, 90% ethanol, 90% acetone (1:1), 90% acetone soaked for 15-20min each (above 4 degrees in the refrigerator), 100% acetone room temperature 15-20min/3 times. The tissue was embedded in the pure acetone and the embedding solution (2:1) at room temperature for 3-4h, or embedded in the pure acetone+embedding solution (1:2) overnight at room temperature, then embed in embedding solution for 2-3h at 37℃. Curing: the tissue was put in the 37℃ oven overnight, in 45℃ oven for 12h, in 60℃ oven for 48h and sectioned into 70 nm slices. The samples then were double stained with 3% uranium-citrate, observed by Jeoljem-1230 (80 kV) and photographed.

Statistical Analysis The experimental data were analyzed by statistical software package. The data were expressed by the mean±standard deviation of the sample (mean±SD). The comparison between the two groups of samples was performed by independent sample *t* test, and the comparison of multiple groups of samples was performed to test the homogeneity of variance. One-way ANOVA was used for comparison. The LSD method was used to compare the variances, and the Dunnet's T3 test was used for the variance. $P<0.05$ was considered statistically significant.

RESULTS

Experimental Animal Situation The 12 eyes of the research rabbits were included in the statistical analysis, since 5 rabbits died of pneumonia, 2 eyes with iatrogenic retinal detachment, 1 eyes with iatrogenic cataract. Results of slit lamp microscope examination: all corneas were transparent within 3wk after operation; conjunctival congestion was obvious on the bulbar conjunctiva and palpebral conjunctiva of the upper and lower eyelid at the first day after operation, and gradually relieved within 2-3d after operation. Conjunctival hyperemia disappeared within 4-7d after operation. Anterior chamber was clear. Pupil was round with good reaction to light. The lens was transparent. Fundus structure was clear, and there was a transient inflammatory reaction in the early stage, which disappeared within 5d after the operation. The filling in the vitreous cavity remained transparent throughout the observation. The retina was flat without retinal detachment and hyperplasia.

Effect of Cross-linked Sodium Hyaluronate Gel on Intraocular Pressure After administration with high molecular weight HA, the full-thickness choroidal structure was observed by optical coherence tomography (OCT). As shown in Figure 1A, there was no significant changes in the thickness of the choroidal subfovea compared with the control group, indicating that this new material has good biocompatibility with eye tissue. Then, transmission electron microscopy was performed to observe the ultrastructure of

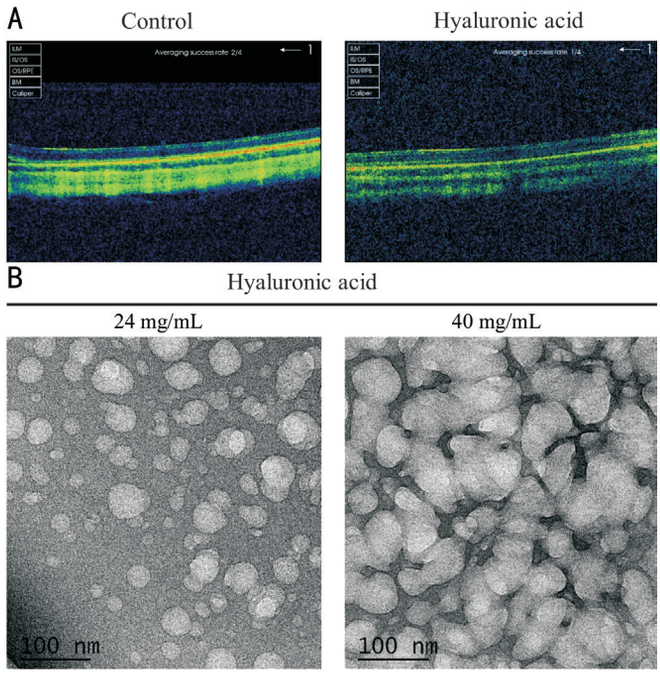

Figure 1 Nanoparticles of HA polymerization was observed under transmission electron microscopy.

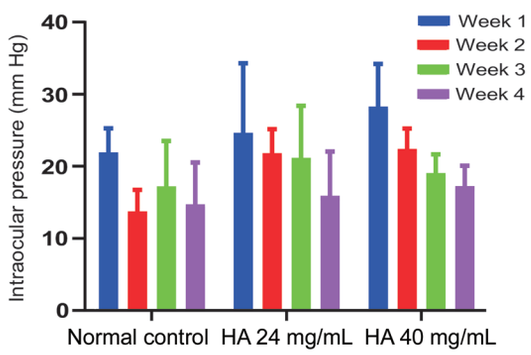

Figure 2 Evaluation of rabbit intraocular pressure (mm Hg) at different time points after surgery in the research group (HA 24 mg/mL, HA 40 mg/mL) and normal control group.

cross-linked sodium hyaluronate gel. As shown in Figure 1B, it was homogeneity nanoparticles, most of which was less than 100 nm. After vitrectomy, cross-linked sodium hyaluronate gel was given in the eyes of rabbits, and intraocular pressure was measured at different time points (Figure 2). Intraocular pressure was increased in the first week after surgery. In research group, comparing with the first week after surgery, the intraocular pressure in HA 24 mg/mL and 40 mg/mL groups were decreased gradually. At week 4, the intraocular pressure was closed to the normal control group ($P>0.05$).

Ophthalmology B-ultrasound Test on Rabbit Eyes In the two different concentrations of sodium hyaluronate group, the ocular B ultrasound investigated the flocculation echoes in the vitreous cavity of eyes (Figure 3), and the retina was flat. In the control group, a small number of vitreous strips in both eyes enhanced echoes, while no abnormalities were observed in the retina.

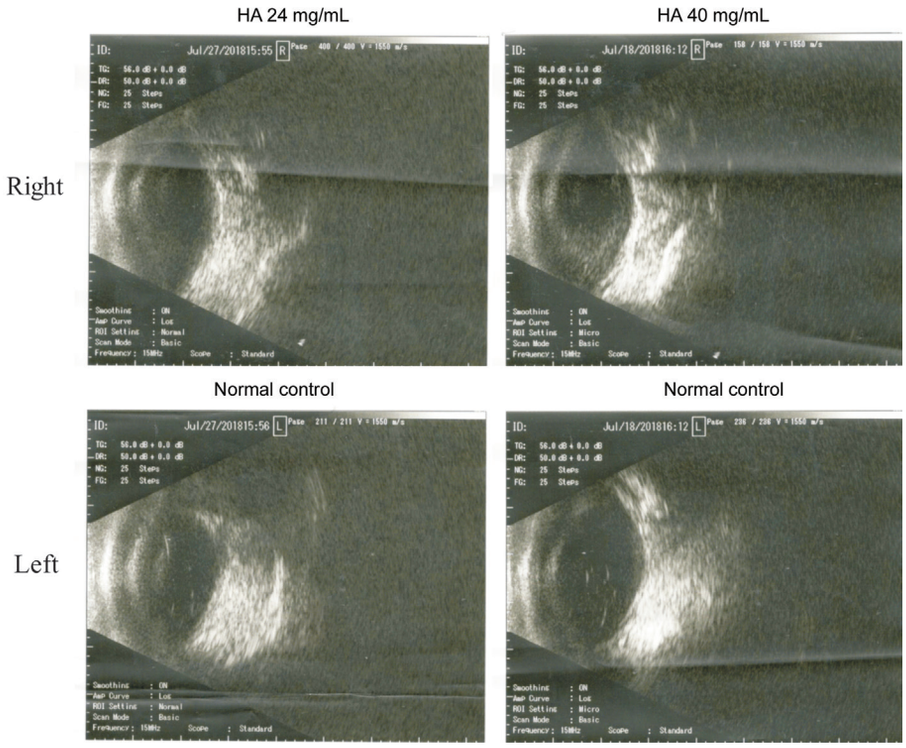

Figure 3 B-ultrasound in rabbit eyes with different concentration of HA materials.

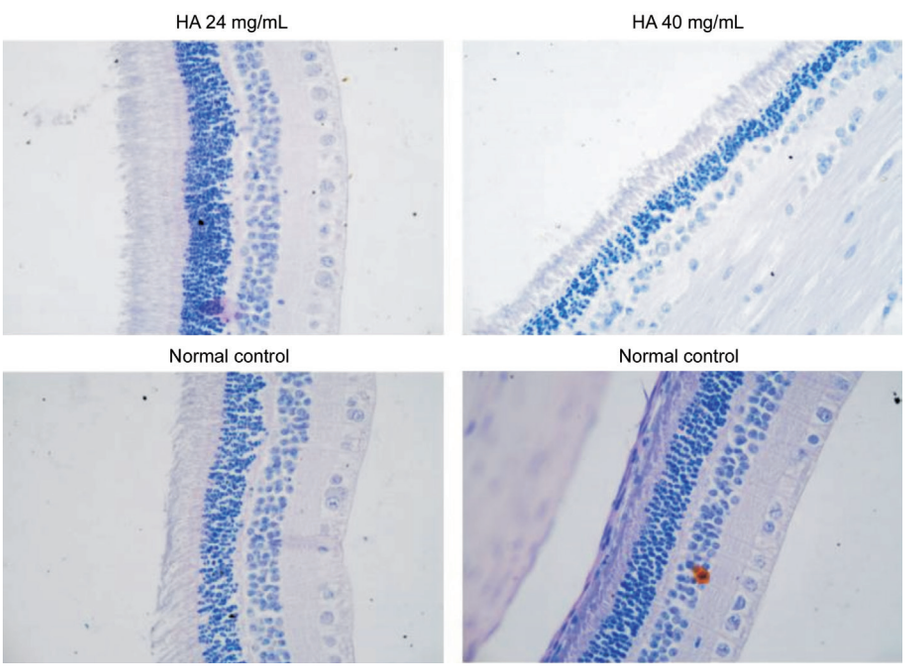

Figure 4 HE staining and physiological structure of rabbit retina was shown after treatment with different concentrations of HA materials Scale bar=50 μm.

Effect of Cross-linked Sodium Hyaluronate Gel on Physiological Structure of Rabbit Retina After HE staining, it can be seen under a light microscope. In the control group, all layers were well-structured and arranged neatly, and the retinal cones and rod cells were arranged normally. In the HA24 mg/mL group, the local arrangement of cone and rod cells was slightly uneven. In the HA 40 mg/mL group, the cones and rod cells were narrowed slightly, and the outer plexus layer of the retina was wider than the other group (Figure 4).

Effect of Filling Materials on Ultrastructure of Rabbit Cells The result of transmission electron microscopy shown that (Figure 5), the mitochondria in the normal control group were normal in morphology with obvious protuberance, and no swelling or fusion was observed. In the research group (24 mg/mL), the structure of the outer segment of the rod-

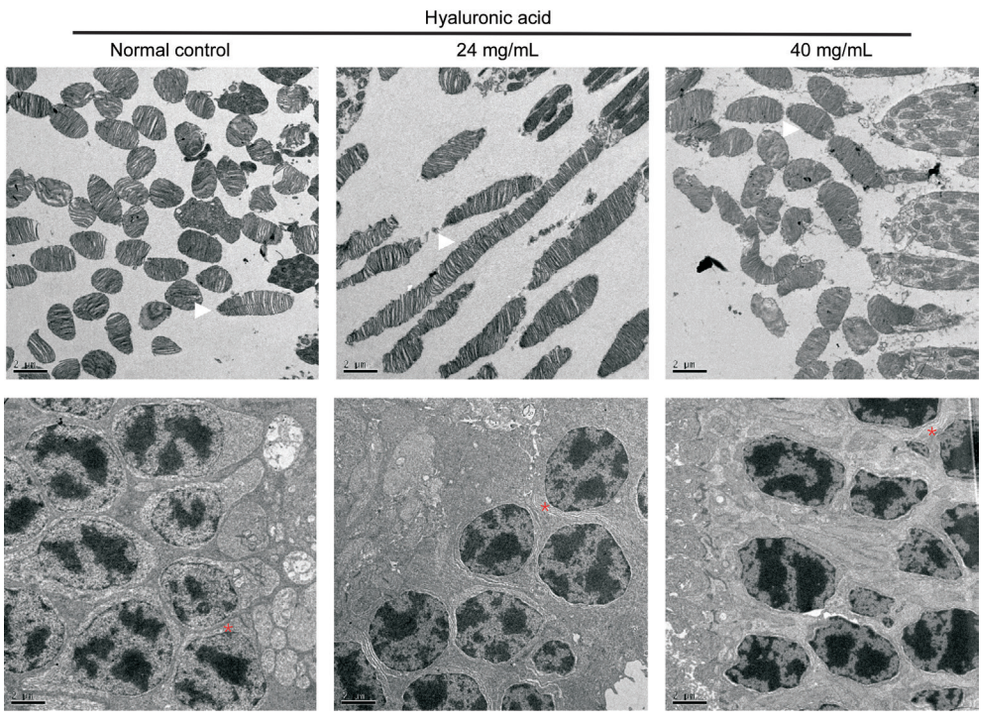

Figure 5 Ultrastructure of rabbit eyes was observed under electron microscopic with different concentrations of HA materials The top column is the typical rod-shaped cell, as shown by the white arrow, the bottom column is the typical main cell, red asterisk indicates the cell endoplasmic reticulum.

shaped cell was complete, and the microfilament microtubules were clear without rupture (Figure 5, upper column). The cytoplasm of the main cell was uniform (Figure 5, lower column). In the research group (40 mg/mL), the structure of the outer segment of the rod-shaped cells was complete, arranged neatly and closely. However, the microtubules were seriously damaged and the ultrastructure was fuzzy. The cytoplasm of the main cell was uniform, the endoplasmic reticulum were not enlarged, nucleation, condensation, dissolution and fragmentation were not observed.

DISCUSSION

At present, the surgical methods for retinal detachment including intraocular gas injection, scleral cerclage, vitrectomy, vitrectomy with silicone oil or inflation gas[11]. These types of surgery can restore the right position of retina, while there are some differences between the operations in terms of complications caused by surgical materials, for example in scleral buckling, eyeball deformation, and worsen myopia would be occur after surgery, resulting in various complications including diplopia, strabismus, anterior segment ischemia, astigmatism, cerclage pain, secondary glaucoma, pressurized material exposure, and even infection. Since the first report of injecting air into the vitreous cavity to repair retinal detachment, people have been looking for an ideal vitreous substitute. After vitrectomy, infusion gas or silicone oil surgery has facilitated the progress of vitreoretinal surgery. As a vitreous filler, silicone oil has been widely used in clinical operations of complex retinal detachment from vitreous body, which greatly improves the cure rate of retinal detachment[12]. In the clinic, the density of the commonly used vitreous substitutes, including SF6, C3F8, silicone oil, and others, are less than water, and the top pressure effect on the lower retinal tear hole is not ideal. After silicone oil or gas injection, the patient must remain in a prone position for a certain period of time. In recent years, in order to solve the problem of lower retinal reattachment, heavy silicone oil has been applied in the clinic. Although it has a good pressure on the lower retina, it may pull the upper retina and form the upper retinal tear in the upright position with the upper retina detached. Therefore, there is an urgent need for a vitreous filler with a specific gravity greater than water, good filling effect, good tissue compatibility, no recipient limitation, and effective compression of retinal tears according to the orientation of the holes. In theory, vitreous substitutes can compress any part of the retinal tears at will, and if it is not toxic to the eye tissue, it may be a long-term vitreous filler[13].

Previous experiments have shown that HA itself is not toxic. It is widely used in ophthalmology and exhibits good clinical biocompatibility[14-15]. However, some studies have revealed that different preparation processes may lead to subtle differences in the biocompatibility of the final product of HA, such as dihydrazide cross-linked hydrogels and ultraviolet cross-linked hyaluronic acid (UV-CHA) hydrogels[16-17]. Hydrogels show improved biocompatibility

after dialysis. Our finding also supports previous studies with variable photo-crosslinked hydrogels that also showed good biocompatibility[14]. Based on these results, UV-CHA hydrogels were chosen as a more suitable vitreous substitute, and only these gels were used for further experiments. The refractive index of a hydrogel is similar to human vitreous body or water, which seems to be a common feature of hydrophilic hydrogels and may contribute to visual rehabilitation. In contrast, silicone oil has a refractive index of about 1.4 and induces a hyperopic shift of about 4 to 6 D after surgery.

For application during vitreoretinal surgery, the cross-linked hydrogel should be injected by needle. Our rheological experiments show that after injection through the 20 gauge needle, the kinematic viscosity is much higher (61-94 Pa·s) than that of silicone oil (approximately 4.85 Pa·s) which has a dynamic viscosity of 5000 cSt and remains higher in kinematic viscosity at low shear rates. The result could be supported by the study from Schramm *et al*[18], in which UV cross-linked N-vinyl-pyrrolidone hydrogels were analyzed and N-vinyl-pyrrolidone is also the component of UV-CHA. The high viscosity of the cross-linked hydrogel is essential for the packing effect and can provide mechanical resistance to proliferative vitreoretinopathy. In addition, high viscosity can avoid turbulence in the area around the retinal hole and prevent re-escape of the retina and cause secondary bleeding into the vitreous cavity. HA in its natural form degrades rapidly in the human eye, whereas cross-linking forms has been successfully used to significantly slow down degradation, it was reported that the UV-CHA biogel remained stable for more than 6wk in the vitreous cavity of rabbits. The stability and persistence of long-term vitreous substitutes in the vitreous cavity is very important[19], especially in the treatment of retinal detachment with markedly proliferative vitreoretinopathy[20]. Past experience of MIRAgel (MIRA, Inc., Waltham, MA, USA) indicates unexpected complications may occur in the body[21].

During the whole observation period, the color of the optic disc and the retinal blood vessels of the different concentrations of the experiment were normal. No disc edema, optic atrophy, retinal necrosis, and no retinal tissue were observed. The HE-stained retinal tissue sections were observed under an optical microscope. The layers of the retina were clearly structured and arranged neatly. No obvious abnormalities in the retinal tissue were observed. It shows that HA polymer material has no damage to the retina. Under the light microscope, the HE stained optic disc slices were observed. After administration with the HA polymer materials in the two research groups, the optic disc structure was intact, and no edema and other damage were observed. It indicates that the HA polymer material can be absorbed along with the moving part of the vitreous body. The results of this study showed that the anterior segment of the rabbit eye was stable within 3mo after the injection of the magnetic fluid in the rabbit vitreous cavity, and no cataract occurred, and no damage was found in the retinal tissue of the fundus. There was no significant difference between the injection of 20 and 40 mg/mL different concentrations of HA cross-linked sodium hyaluronate gel. This study suggests that cross-linked sodium hyaluronate gel appears to have good stability and transparency in the vitreous cavity for at least 6wk. In our animal experiments, the injection of cross-linked sodium hyaluronate gel alone seems to be an expected method for successful treating of iatrogenic retinal detachment. The above data confirmed the good biocompatibility of the cross-linked sodium hyaluronate gel and may be a valuable alternative to long-term vitreous replacement. In addition, in the future, it may be considered to add the drug to the hydrogel during the cross-linking process to provide the clinician with a sustained release system. This approach may open up new options to prevent and treat retinal detachment.

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