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

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Research article

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Abstract

Purpose: To observe the effects of cross-linked hyaluronic hydrogel on retina struct and intraocular biocompatibility in rabbits. Methods: the polymer-derived hyaluronic acid which was formed by UV light cross-linked with N-vinyl-pyrrolidone. Free hyaluronic acid levels were detected by spectrophotometric method. Vitrectomy was then performed in the rabbits, and then cross-linked hyaluronic acid hydrogels of 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. The eyeballs were fixed for HE staining, and the polymer materials were observed under electron microscopy. Results: The synthetic hydrogel present a transparent substance with a refractive index similar to that of the human vitreous body, which also had sufficient viscosity and elasticity for intraocular usage by rheological measurements. The results of the free hyaluronic acid test showed that the hydrogel had little degrade within one month. The results of the histology, intraocular pressure, B-ultrasound and retinal ultrastructure suggested that cross-linked hyaluronic acid hydrogel had superior tissue biocompatibility intraocular for six weeks. Conclusions: Hyaluronic acid-based cross-linked biopolymers had good biocompatibility in rabbit, which also shown promising potential as vitreous substitutes in clinical practice.

Background

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 can 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] .Therefore, vitreous tamponades with good biocompatibility, stable properties, and no recipient limitation, should be developed to effectively press the retinal tears with different orientations [4] .

Natural hydrophilic polymers, such as hyaluronic acid (HA) polymers that meet these needs are found by the researchers. 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 [5] . 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 [6] . Chirila and Hong et al. 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 [7, 8] . In this study, two different concentration of hyaluronic acid polymer materials obtained by UV cross link were compared in optical and rheolgoical properties, and their effect of long-term biostability and histocompatibility in rabbit eyeballs were investigated.

Methods

Materials.

HA from the cockscomb was selected and had a molecular weight of 1-4 × 10^6 for the synthesis of crosslinked hydrogels. Cross-linked hyaluronic acid was produced by Hangzhou Xiehe Medical Biological Preparation Co., Ltd., with two specifications of 24 mg/ml (batch No. S29171002) and 40 mg/ml (Batch 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.

Experimental animals and grouping.

All procedures in this study complied with the ARVO statement for the use of animals in research. 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 1.8-2.2kg, male, and have no anterior segment lesions in both eyes. The right eye was involved in the experimental group while the left eye in the control group. After animal modeling, the experimental animals were divided into two groups: A) cross-linked sodium hyaluronate gel 24 mg / ml; B) cross-linked sodium hyaluronate gel 40 mg / ml.

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.

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 mmHg. The intraocular balance fluid is exchanged from the body. 1.0-1.2 ml of two different concentrations of cross-linked hyaluronic acid were slowly injected from the perfusion tube into the vitreous cavity in the experimental groups, 24 mg/ml for experimental group 1 and 40 mg/ml for group 2. The intraocular pressure was 5-10 mmHg detected after surgery. Tobramycin eye drops were applied 3 times/d for 3-5 days. Compound tropicamide sputum were applied for mydriasis, 2 times/d, for 3 days.

Clinical observation was made at different time points after the surgery: left and right corneas were observed at l d, 1 weeks, 2 weeks, 3 weeks, 1 months and 3 months after the surgery. B-ultrasound and pupil dilation were taken for complications such as lens opacity, vitreous hemorrhage, endophthalmitis and others.

Pathological examination.

The experimental animals were sacrificed by air embolization 3 months after the surgery, and the eyeballs were removed (12 effective eyeballs in the experimental group). The retinal tissue (10mm× 10mm×1mm) 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 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, 15 min each, and in 100% xylene for 2 times, 15 minutes each. Embedding: the tissue was soaked in 56 degrees paraffin wax for 4 times, 30 min 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 (TEM).

TEM is performed to observe the ultrastructe changes of. Sample preparation: the tissue was rapidly cut into 1×1 mm^3 size, fixed with 2.5% glutaraldehyde (containing 0.1 m phosphate buffer) for more than 2 h, rinsed with 0.1M phosphoric acid solution for 15 min/3 times, fixed with 1% osmic acid for 2-3 h, followed by gradient dehydration: 50%, 70%, 90% ethanol, 90% acetone (1:1), 90% acetone soaked for 15-20 min each (above 4 degrees in the refrigerator), 100% acetone room temperature 15-20 min/3 times. The tissue was embedded in the pure acetone and the embedding solution (2:1) at room temperature for 3-4 h, or embedded in the pure acetone + embedding solution (1:2) overnight at room temperature, then embed in embedding solution for 2-3 h at 37 degree celsius. Curing: the tissue was put in the 37 degree celsius oven overnight, in 45 degree celsius oven for 12 h, in 60 degree celsius oven for 48 h 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 (± S). 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.

12 eyes of the experimental 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 3 weeks 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 2-3 days after operation. Conjunctival hyperemia disappeared within 4-7 days 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 5 days 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.

Transmission electron microscopy showed that cross-linked sodium hyaluronate gel was good in homogeneity and nanoparticles of less than 100 nm were formed (Fig. 1). After vitrectomy, cross-linked sodium hyaluronate gel was given in the eyes of rabbits, and intraocular pressure was measured at different time points (Fig. 2). Intraocular pressure was increased in the first week after surgery which may be caused by surgery and the stimulation of fillers. In experimental group, the intraocular pressure in HA 24mg/ml group was decreased at 4 weeks after surgery comparing 1 week after surgery, which indicated the stimulation could be eliminated gradually. While in HA 40 mg/ml the intraocular pressure was increased gradually, which was eliminated in at week 8 finally (data not shown), which indicated the high concentration of HA may cause the sustain upregulation of intraocular pressure than low concentration. In the control group, the intraocular pressure in most of the rat eyes could be decreased at week 2 (P 0.05), while some had the fluctuation which may be caused by the surgery.

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 both eyes (Fig. 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.

Effect of cross-linked sodium hyaluronate gel on the physiological structure of rabbit retina.

Light microscopy: Group A: The local arrangement of cone and rod cells was slightly disordered, and the outer plexus layer of the retina was wider than that of group B and C. The retinal cone and rod cells were arranged normally. Group B and C: The cones and rod cells were arranged in a disordered manner, and the outer layer of the retina was narrowed. The retinal cone and rod cells was arranged normally. Group D: All layers were well-structured and arranged neatly, and the retinal cones and rod cells were arranged normally (Fig. 4).

Effect of filling materials on ultrastructure of rabbit cells.

Transmission electron microscopy: the experimental group (24 mg/ml): all 10 layers' structure of the retina were complete and the cells in each layer had no obvious degeneration and necrosis. The outer plexiform layer was not thinned, and there was no synapse reduction (Fig. 5) . In the experimental group (40 mg/ml): all 10 layers' structure of the retina were complete and the cells in each layer had no obvious degeneration. The mitochondria and endoplasmic reticulum were not swollen, no nuclear pyknosis, dissolution, fragmentation and etc., the ribosome of the outer nuclear layer was increased and aggregated. The outer plexiform layer became thinner and synapse was decreased (Fig. 5).

Discussion

At present, the surgical methods for retinal detachment including intraocular gas injection, scleral cerclage, vitrectomy, vitrectomy with silicone oil or inflation gas. 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 occour after surgery, resulting in various complications incluing 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. In the clinic, the commonly used vitreous substitutes SF6, C3F8, silicone oil and others are less than water in specific gravity, 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.

Hydrogel glass substitutes based on cross-linked hyaluronic acid have many theoretical advantages in terms of biocompatibility and filler properties because they mimic the properties of natural human vitreous bodies. Cross-linked hyaluronic acid hydrogels combine many vitreous properties such as optical clarity, high viscosity, long-term stability, and good biocompatibility. The study found that the vitreous viscosity of healthy people is 300-2000 cP (0.300-2Pa·s) [9, 10] . The viscosity of the cross-linked hyaluronic acid hydrogel in this study is many times than that of vitreous body, and a complete packing effect will make it become an ideal vitreous substitute. In addition to this packing effect, it is mentioned in the literature that its high expansion factor for good reattachment of the retina after surgery is advantageous. However, significant swelling of the material within the vitreous cavity may cause an increase in intraocular pressure. In this study, only a slight swelling appears by the use of the cross-linked hyaluronic acid hydrogel. This study shows that hydrogels like this can replace hydrophobic silicone oil as a long-term vitreous substitute in principle. Currently, only silicone oil can be used as a long-term vitreous substitute. Silicone oils have a number of disadvantages when used as a vitreous alternative, including the risks of secondary glaucoma, cataract formation, hyperopia shifting, and the need for additional surgery to remove away. In addition to transparency, stability, sufficient viscosity and biocompatibility, another practical advantage of hydrogels is that their hydrophilic nature is opposite to that of hydrophobic silicone oils. These properties facilitate the complete filling of the vitreous cavity with good filling effect. Due to their hydrophilic nature, hydrogels can be used as a delivery system for sustained drug release, as described in various other hydrogels and biopolymers [11] . Hydrophilic vitreous substitutes such as hydrogels can be loaded with drugs for extended release into the vitreous cavity [12] .

Previous experiments have shown that HA itself is not toxic. It is widely used in ophthalmology and exhibits good clinical biocompatibility [13, 14] . Experiments with rpells demonstrate the relative differences in biocompatibility between dihydrazide cross-linked hydrogels and uv-cha hydrogels [15] . Although UV-CHA hydrogels did not show any signs of cytotoxicity or adverse effects on cell proliferation, experimental hydrogels cross-linked with ADH-HA did not completely ban harmful biological effects. Another finding of the ADH-HA cross-linked experimental hydrogels was a slightly increased cellular mitochondrial activity observed in the MTT assay as confirmed by the Alamar Blue assay. This finding may be critical for the potential enhancement of proliferative vitreoretinopathy. These results are slightly different from those obtained by Su et al., which showed that similar cross-linked ADH-HA hydrogels have no toxic effects on RPE cells. In fact, they add more steps to the reaction before cross-linking HA, so toxic substances can be removed in advance. However, in any case, it seems likely that after cross-linking, the ADH-HA hydrogel retains some cross-linking agent that induces the observed cytotoxic effects, which underscores the importance of careful washing. Hydrogels show improved biocompatibility after dialysis.

This finding also supports previous studies with variable photo-crosslinked hydrogels that also showed good biocompatibility. Based on these results, UV-CHA hydrogels were carried out 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 [16] . In contrast, silicone oil has a refractive index of about 1.4 and induces a hyperopic shift of about 4D to 6D 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 D'Errico et al [17] , 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. Our in vitro degradation rate was only 10% in the first 4 weeks and much slower in the next few months. The hydrogel remains stable without losing its properties. However, it must be remembered that the environment in the human vitreous body is different from the in vitro conditions in these experiments. Hong et al. propose that true degradation may be much more faster [7, 8] . However, the UV-CHA biogel remained stable for more than 6 weeks in the vitreous cavity of rabbits. The stability and persistence of long-term vitreous substitutes in the vitreous cavity is very important, especially in the treatment of retinal detachment with markedly proliferative vitreoretinopathy. Past experience of MIRAgel (MIRA, Inc, Waltham, MA) indicates unexpected complications may occur in the body [18] .

This study showed that the anterior segment of the rabbit eye was stable within 3 months and no cataract occurred after injection of the HA polymer material into the vitreous cavity. 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. At 1 week after operation, different degrees of HA polymer nanoparticles were observed on the left and right vitreous myopic disc sides, suggesting that the HA polymer material can float and move randomly in the vitreous cavity. At 1 month and 3 months after operation, the HA polymer materials of the two experimental groups were reduced, 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 3 months 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 mg/ml 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 6 weeks. 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.

Abbreviations

HA: hyaluronic acid (HA); TEM: Transmission electron microscopy; UV: Ultraviolet; HE staining: Hematoxylin-eosinn staining RPE cells

Declarations

Ethics approval and consent to participate

The authors have no ethical conflicts to disclose.

Authors' Contributions

GY designed and performed the experiment and was the major contributor in writing the manuscript. CK analyzed and interpreted the data. WY contributed to the design the work and to article revision. GXH and ZA made substantial contributions to the conception and design of the study. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Medical Ethics Committee of Ningbo eye hospital and supervised throughout the process.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

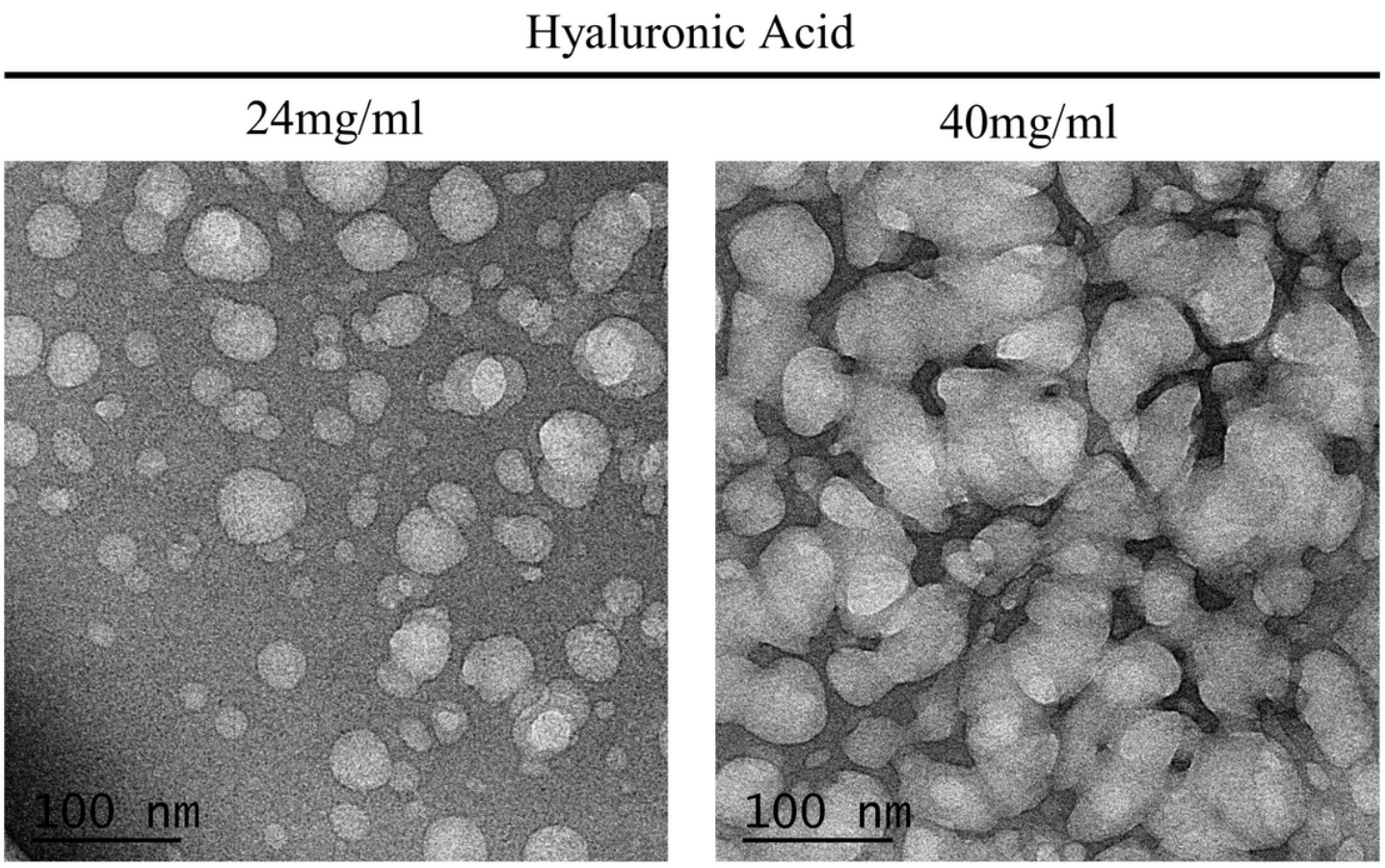

Figure 1

Nanoparticles of hyaluronic acid polymerization was observed under transmission electron microscopy.

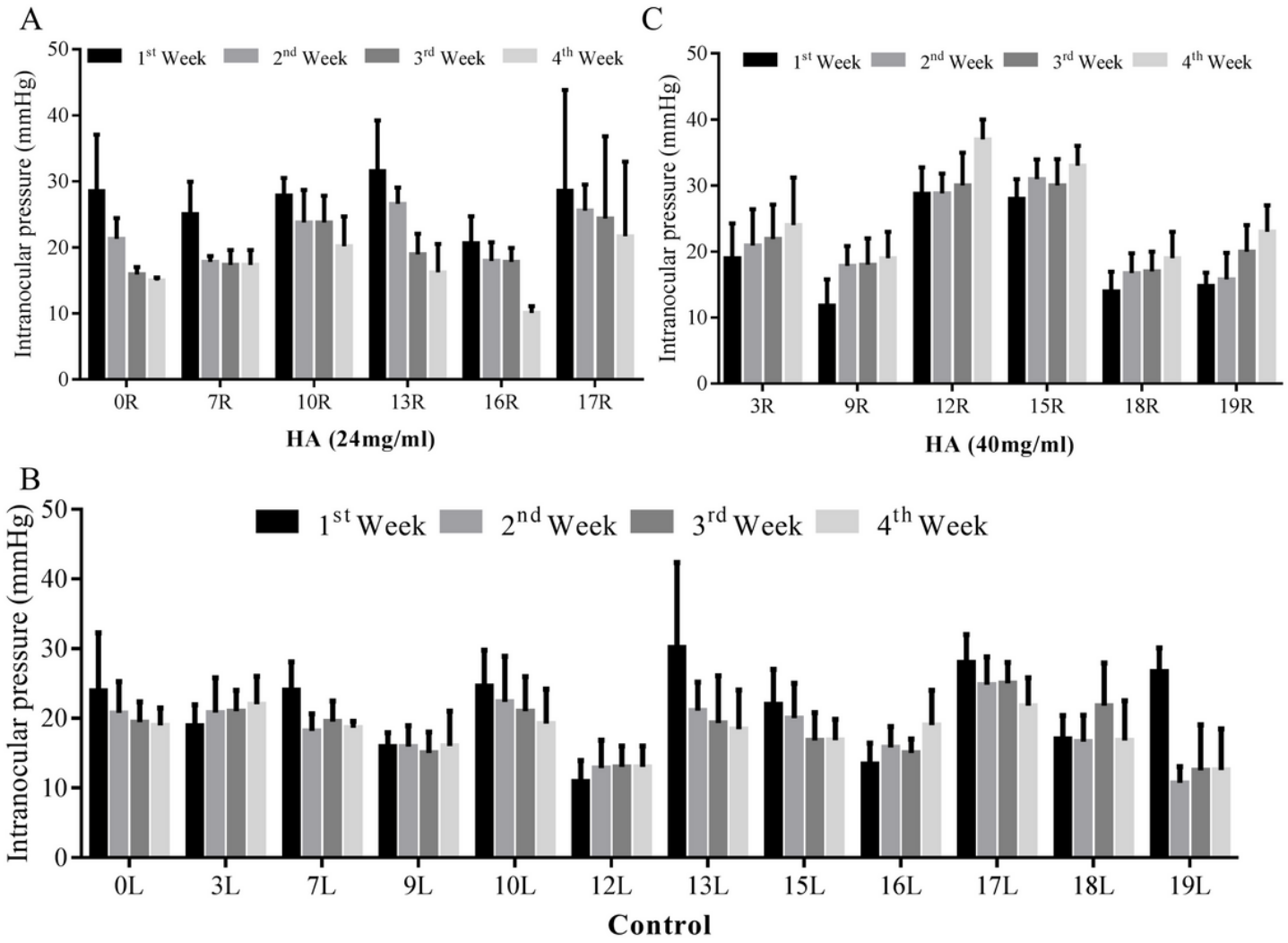

Figure 2

Comparison of rabbit intraocular pressure (mmHg) at different time points before and after surgery in the experimental group (24mg/ml and 40mg/ml, control group = 0mg/ml).

Figure 3

B-ultrasound in rabbit eyes with different concentration of hyaluronic acid materials.

Figure 4

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

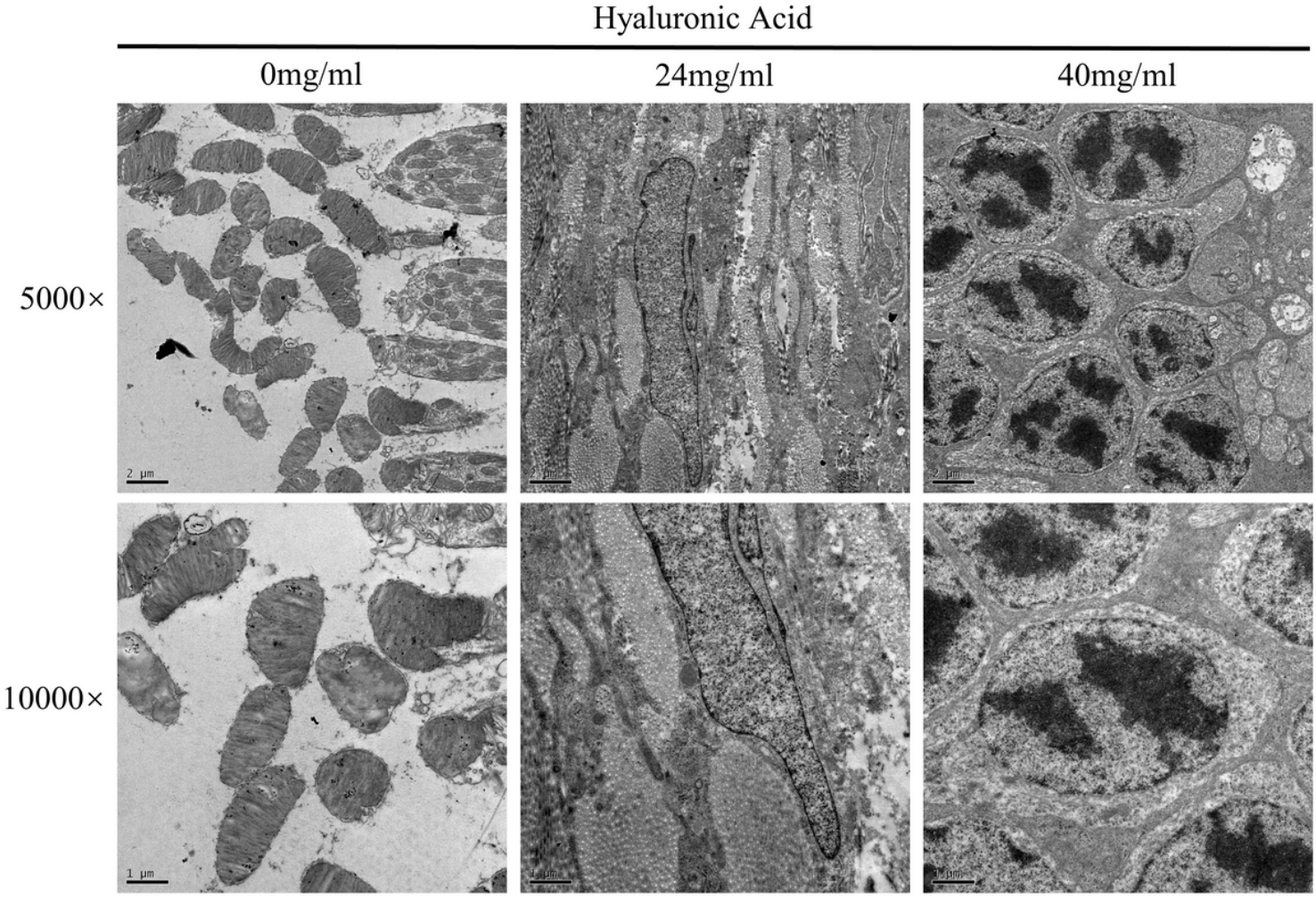

Figure 5

Ultrastructure of rabbit eyes was observed under electron microscopic with different concentrations of hyaluronic acid materials.

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