Article

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Patching Retinal Breaks with Chitosan for Retinal Detachment in Rabbits

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Background: Rhegmatogenous retinal detachment (RRD) is caused by one or more full-thickness retinal breaks. The current RRD treatments have several drawbacks. Chitosan is one of the most commonly used natural polymers for wound healing and has been demonstrated to be biodegradable, biocompatible, non-toxic, bioadhesive, and bioactive. This study aimed to determine the reliability and effectiveness of chitosan for sealing retinal breaks in rabbits.

Methods: Eighteen blue purple rabbits were randomly divided into three groups: chitosan (n = 6), RRD (n = 6), and control (n = 6). The RRD model was established using vitrectomy, making retinal holes, and subretinal fluid injection in the RRD and chitosan groups. One week after the establishment of the model, chitosan was applied within the range of the holes in the chitosan group, and the vitreous body was filled with perfusion fluid. Except the chitosan treatment, the RRD group underwent the same procedure. Intraocular pressure (IOP) measurement, fundus photography, B-mode ultrasound, optical coherence tomography (OCT), histology, and enzyme linked immunosorbent assay (ELISA) were performed.

Results: Retinas of all eyes in the RRD group were detached, whereas those of all eyes in the chitosan group remained attached. The concentrations of epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and IL-8 in the vitreous fluid of the RRD group were significantly higher than those of the control group ($p < 0.05$). Furthermore, the concentrations of EGF, FGF-2, TGF-β, and VEGF in the vitreous fluid of the chitosan group were higher compared to those of the RRD group ($p < 0.05$), whereas the concentrations of IL-6 and IL-8 were lower ($p < 0.05$).

Conclusions: Chitosan may be a reliable method for sealing retinal breaks. Moreover, chitosan can maintain high levels of growth factors and reduce inflammatory factors in the vitreous, which may reduce and delay the death of retinal cells and help restore visual function after retinal repositioning.

Keywords: rhegmatogenous retinal detachment; chitosan; intraocular cytokines; vitrectomy; reliability

Introduction

Retinal detachment (RD) is the separation of the retinal nerve fiber layer and retinal pigment epithelium (RPE), and rhegmatogenous retinal detachment (RRD) is caused by one or more full-thickness retinal breaks [1]. In most cases, RRD is treated with scleral buckling; vitrectomy with vitreous tamponade by air, gas, or silicone oil combined with cryotherapy; or laser photocoagulation [2]. However, in some cases, RRD failure is often due to the inability to maintain retinal rupture closure and proliferative vitreoretinopathy (PVR) caused by the migration of RPE cells through the retinal rupture [3]. It seems reasonable to repair retinal breaks with glue or film, which can prevent vitreous fluid from flowing into the subretinal space through the retinal breaks and prevent RPE cells from dispersing into the vitreous cavity [4]. This sealant can also prevent the filling with gas or silicone oil and avoid a face-down position for an extended period after surgery [5].

Therefore, in experimental retinal detachment models or clinical cases, adhesives such as such as fibrin glue, sodium hyaluronate/carboxymethyl cellulose absorbable membrane, mussel protein, cyanoacrylate, and transforming growth factor β and polysiloxane have been used to seal retinal breaks [6–9]. However, each method has its disadvantages, including potential ocular toxicity, difficulty of intraocular administration, weak adhesion, inflammatory reaction, and granuloma tissue reaction [6,8]. Due to these shortcomings, adhesives have not yet become the standard method for treating rhegmatogenous retinal detachment [6]. There is an urgent need for a better material that is nontoxic, effective, and convenient for sealing retinal tears.

Chitosan is one of the most commonly used natural polymers for wound healing [10]. It is a linear copolymer of D-glucosamine and N-acetyl-D-glucamine [11]. Chitosan, a polymer presented in the cell walls of fungi and the exoskeleton of crustaceans, is a relatively cheap and abundant material [12]. Most importantly, it has been demon-

strated to be biodegradable, biocompatible, non-antigenic, non-toxic, bioadhesive, anti-diplococcal, biologically active, and capable of hemostasis [12–14]. In addition, chitosan accelerates wound healing by recruiting growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, transforming growth factor β (TGF-β), and vascular endothelial growth factor (VEGF) [15]. Chitosan also promotes tissue granulation and accelerates wound healing by recruiting inflammatory cells, such as polymorphonuclear leukocytes (PMN) and macrophages, to the wound site [16]. A study showed that chitosan-gelatin blends could be used as biodegradable materials for scleral buckling surgery [17].

This study aimed to evaluate whether and how chitosan repairs retinal rupture in rabbits with RD.

Materials and Methods

Experimental Animal

Eighteen blue purple male and female rabbits weighing 2.0–2.5 kg were purchased from Beijing Tiantan Biological Products Co., Ltd. Beijing, China. They were randomly divided into chitosan, RRD, and control groups, with six rabbits in each group.

Experimental Rhegmatogenous Retinal Detachment

All animals in the chitosan and RRD groups received general anesthesia (ketamine 1 mg/kg intramuscular injection + diazepam 0.1 mg/kg intramuscular injection) and topical anesthesia (50 g/L alcaine). The right eye was selected as the operating eye for the closed three-incision vitreoretinal surgery. The vitreous body was fully excised, a retinal hole with a diameter of 1 papillary diameter (PD) was made at 2 PD below the optic disc, and retinal detachment was caused by injecting perfusion fluid under the retina. One week later, the chitosan group underwent closed three-incision vitreoretinal surgery; 0.1 mL chitosan was injected into the hole using a syringe and evenly distributed throughout the hole area. Air in the vitreous humor was replaced with a balanced salt solution. The RRD group underwent the same procedure as the chitosan group, except for the application of chitosan. All scleral ostia and conjunctival wounds were sutured using 8-0 suture. At the end of the surgery, an antibiotic ointment was applied to the eyes. The eyes of animals undergoing surgery were administered 0.1% tobramycin and 1.5% levofloxacin three times a day after surgery.

Clinical Examination

Pupil dilation in rabbits was observed using slit-lamp microscopy and indirect fundoscopy before and 1, 3, and 7 days after surgery. A rebound tonometer (Icare® Pro, Helsinki, Finland) was used to measure intraocular pressure (IOP) before surgery and at 1, 3, and 7 days after surgery. Fundus photography (CE-1, Canon Corporation, Tokyo, Japan), optical coherence tomography (OCT) (SPECTRALIS OCT, Heidelberg Corporation, Heidelberg, Germany), and B-mode ultrasonography (Compact Touch, Guangtai Company, Auvergne, France) were used to evaluate the retinal state. IOP was evaluated using repeated-measures analysis of variance.

Histology

On day 3 and 7 after operation, the rabbits were euthanized with excessive pentobarbital. Their eyes were removed for histological analysis. After eyeball removal, all eyes were fixed in a 2% paraformaldehyde and 2.5% glutaraldehyde solution, and slices were dewaxed with xylene and ethanol at high to low concentrations. The sections were stained with hematoxylin for 5 min, incubated with 1% hydrochloric acid alcohol for 3 s, and stained with eosin for 3 min. Subsequently, the slices were dehydrated using ethanol and xylene at concentrations ranging from low to high. The paraffin embedded retinal tissue section was 5 μm. Sections were fixed with neutral gum and photographed using an optical microscope (M205, Leica Microsystems, Germany).

Enzyme Linked Immunosorbent Assay (ELISA)

On day 7 after the chitosan filling operation, vitreous fluid samples were collected from the vitreous cavities of all rabbits. Specifically a 1 mL syringe was used to penetrate the vitreous cavity below the temporal part 4 mm behind the upper nasal limbus, extract 0.5 mL of liquefied vitreous fluid, and freeze the sample at –80 ℃. Concentrations of EGF, FGF-2, TGF-β, VEGF, interleukin 6 (IL-6), and IL-8 were determined by ELISA (D6050, Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Before the test, the kit and specimen were placed at room temperature for 30 min, and the standard and sample wells were set. Then, 50 μL of the standard at different concentrations was added to the standard wells and 50 μL of the testing sample to the sample wells; the blank well was not filled. Horseradish peroxidase labeled antibody (100 μL) was added to each well. The reaction well was sealed with a sealing plate membrane and incubated in a 37 ℃ water bath or thermostat for 60 minutes. The liquid from the well was discarded, the well was dried using absorbent paper, and each well was filled with detergent (350 μL) and left to stand for 1 min. The detergent was shaken off, the wells were dried with the absorbent paper, and the washing was repeated five times. Next, the liquid in the well was completely shaken off, 50 mL substrate A and 50 mL substrate B was added into each well, and they were incubated in the dark at 37 ℃ for 15 min. Finally, 50 μL termination solution was added to each well, and the optical density (OD) value of each well was measured at 450 nm using a spectrophotometer (DU-530; Beckman Instruments Inc., Fullerton, CA, USA) within 15 min.

Table 1. The IOP of each group.

Time	Control	RRD	Chitosan	$p1$	$p2$	$p3$
1 d	14.05 ± 0.61	8.77 ± 0.29	14.27 ± 0.51	<0.0001	<0.0001	0.51
3 d	14.05 ± 0.52	8.58 ± 0.45	14.3 ± 0.47	<0.0001	<0.0001	0.40
7 d	13.93 ± 0.55	8.17 ± 0.24	14.15 ± 0.47	<0.0001	<0.0001	0.47

Data are presented as the mean ± SD (n = 6). $p1$, RRD vs. control; $p2$, chitosan vs. RRD; $p3$, chitosan vs. control.

IOP, intraocular pressure; RRD, rhegmatogenous retinal detachment; d, day.

Statistical Analysis

Data are shown as the mean ± standard deviation. Statistical analyses were performed using SPSS (version 19.0; SPSS, Chicago, IL, USA). The differences between groups or pre- and postoperatively were analyzed using a paired *t*-tests or repeated-measures analysis of variance. p was set at <0.05.

Results

Slit Lamp Examinations

All eyes in the RRD group showed retinal detachment on day 1 post surgery. No laser, cryotherapy, or gas tamponade was used during the surgical procedure. Retinal reattachment was observed in all eyes in the chitosan group on days 3 and 7 postoperatively. On days 1 and 7 days after surgery, a small number of water cells, a slight flash, and no lens opacity were observed in the anterior chamber of all eyes.

Fundus Examination, B-Mode Ultrasound, and OCT Findings

Fundoscopic examination revealed that none of the eyes in the chitosan group exhibited RD during the entire observation period. However, three eyes in the RRD group showed retinal detachment on days 3 and 7 after surgery. During the fundus examination on day 3 after surgery, the polyethylene glycol (PEG) sealant was observed at the retinal rupture site; however, it could not be observed after seven days (Fig. 1A–D). B-mode ultrasonography of the eyes after surgery revealed that the retinas in the RRD group were widely detached. In the chitosan group, the retina bulged slightly three days after surgery; however, no retinal detachment was observed after seven days (Fig. 1E–H). Postoperative OCT revealed that the retina in the RRD group detached over a large range. In the chitosan group, the edge of the retinal rupture was lifted three days after surgery; however, it was attached and flattened seven days later (Fig. 1I–L).

Intraocular Pressure

During the entire observation period, the IOP in the RRD group was significantly lower than that in the control and chitosan groups ($p < 0.05$). However, there was no significant difference between the IOP in the eyes of the control and chitosan groups ($p > 0.05$) (Table 1).

Histology

Fig. 2A shows the normal morphology and structure of the retina in the control group upon histological examination. All eyes in the RRD group showed complete RD on days 3 and 7 after operation (Fig. 2B), whereas no RD was observed in any eyes of the chitosan group (Fig. 2C,D).

Expression of Cytokines and Growth Factors in the Vitreous Fluid

We measured the levels of cytokines and growth factors (EGF, FGF-2, TGF-β, VEGF, IL-6, and IL-8) in the vitreous fluid. The levels of EGF, FGF-2, TGF-β, VEGF, IL-6, and IL-8 were significantly higher in the control group ($p < 0.05$). Compared with the RRD group; the chitosan treatment further enhanced the expression of EGF, FGF-2, TGF-β, and VEGF, and decreased the expression of L-6 and IL-8 ($p < 0.05$) (Fig. 3).

Discussion

During surgical treatment of RRD, effective hole closure is a key step for the success of the operation. Hole closure methods have explored previously [1]. Currently, the most commonly used methods are external scleral condensation and intraocular laser photocoagulation, which can stimulate the choroid around the retinal hole to produce an inflammatory reaction and form scar adhesions of the pigment epithelium and neuroepithelium, thus achieving retinal hole closure [18]. However, these methods are invasive and can seriously damage the pigment epithelium, increase the dissociation of pigment cells, and increase the probability and severity of PVR. They also destroy the neuroepithelium, weakening or even losing the light sensitivity and signal transmission function of the retina, thus causing the expansion of absolute dark spots or the formation of relative dark spots around the retinal hole area [19]. In addition, neither condensation nor photocoagulation can promote the expected scar adhesion for retinal detachment caused by macular holes and choroidal defect holes in highly myopic eyes due to choroidal atrophy in the hole area; for this reason, such retinal detachment is difficult to reset and relapses easily [20].

Because of the important above-mentioned properties such as biocompatibility, biodegradability, hemostatic and anti-infective activity, and the ability to accelerate

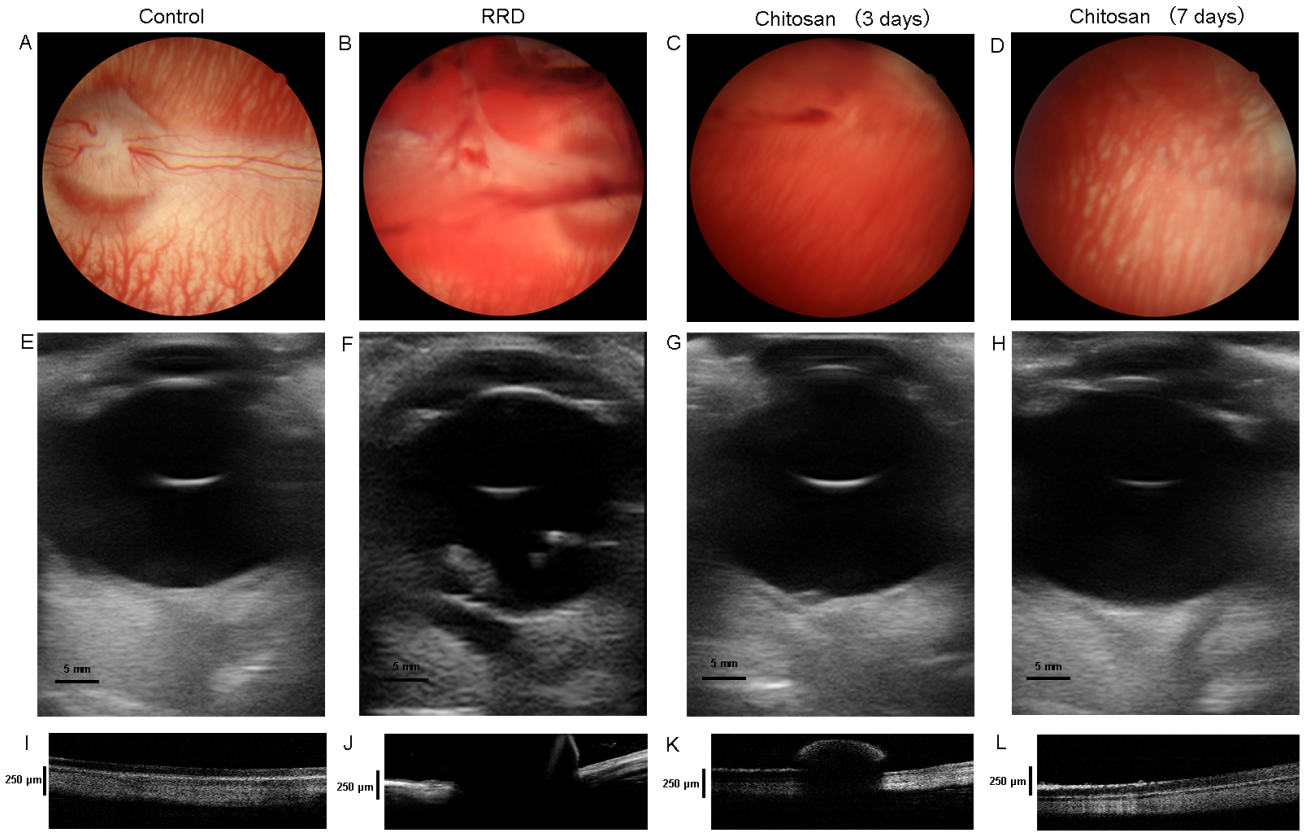

Fig. 1. Fundus examination (A–D), B-mode ultrasound (E–H) and OCT findings (I–L) (n = 6). Chitosan (day 3): chitosan group on day 3 after operation, Chitosan (day 7): chitosan group on day 7 after operation.

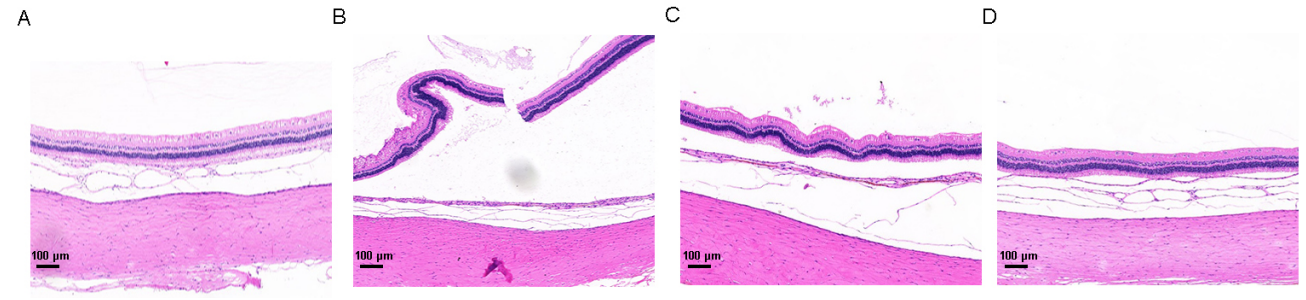

Fig. 2. Histological examination (n = 6). (A) Control group. (B) RRD group. (C) Chitosan group on day 3 after operation. (D) Chitosan group on day 7 day after operation.

wound healing, chitosan has attracted the attention of many researchers for use as a wound dressing [21]. In this study, we demonstrated that chitosan cured retinal tears in rabbits with RRD. Chitosan successfully closed retinal ruptures during surgery and did not require intraocular gas filling. In animal models, materials such as sodium hyaluronate/carboxymethyl cellulose absorbable membranes and hydrogel tissue adhesives can successfully seal retinal rupture and repair RRD. However, because air has been concurrently chosen to fill the vitreous cavity during surgery, it is not clear whether these materials will seal retinal tears and repair RRD immediately after application [9,22]. From a practical point of view, the closure of RRD breaks with chitosan is advantageous for patients, because it eliminates the need for them to maintain a downward position after operation, and can also avoid blurred vision caused by air or gas tamping. Although intraocular gas was not required to maintain retinal reattachment in this study, whether this is also applicable to human clinical practice requires further research.

Growth factors are polypeptides that control cell growth, differentiation, and metabolism, and regulate tissue repair [23]. Although they are present in small amounts, they have a strong impact on wound repair. The use of growth factors to accelerate wound healing is a promising therapeutic strategy for chronic wounds [24]. Several pep-

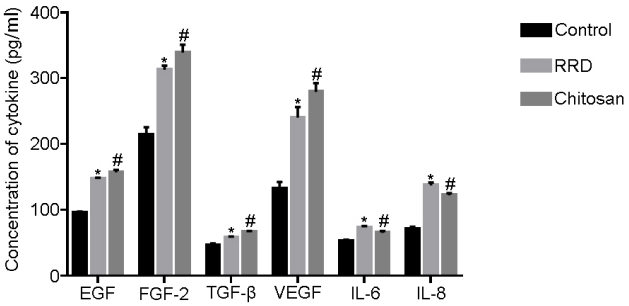

Fig. 3. Expression of cytokines and growth factors in the vitreous fluid (n = 6). *Indicates RRD vs. Control: $p < 0.05$, #Indicates Chitosan vs. RRD: $p < 0.05$.

tide growth factors, such as EGF, platelet derived growth factor (PDGF), FGF, and TGF-β, have been demonstrated to accelerate cell proliferation and extracellular matrix synthesis. Several studies have reported that EGF increases the rates of cell proliferation, growth, and tissue regeneration [25]. Epidermal growth factor (EGF) produced by macrophages and keratinocytes plays an important role in wound closure [25]. Activator protein-1 (AP-1) and STAT-3 serve as EGF receptor signals. Some researchers have found that wound healing is delayed if a receptor signal is absent [26]. Brem *et al*. [27] recently found that the level of EGF secreted by keratinocytes on the wound surface decreases with delayed healing, which may be the cause of wound nonunion. Therefore, if the EGF on the cell surface is reduced, epidermal growth factor receptor (EGFR) signal transmission is inhibited, leading to chronic wounds [27]. FGF plays an important role in the epithelization stage of wound healing. Some studies have found that if the FGF receptor is inhibited, fibroblasts show excessive keratinization [28,29]. FGF is not only a new angiogenic factor, but also a factor that can stimulate the migration and diffusion of fibroblasts [30–32]. The body requires FGF to migrate fibroblasts and promote wound healing [30–32]. In the process of wound healing, many growth factors are involved in the regulation, among which TGF-β is an essential growth factor. A series of signal molecules composed of TGF-β can regulate various life activities of cells, such as cell proliferation, differentiation, adhesion, migration, and apoptosis. They play important roles in the development of organisms and various processes [33–35]. Some scholars found that TGF-β can have a chemotactic effect on neutrophils, fibroblasts, and other cells that play an important role in wound healing [36]. In this experiment, we found that the expression levels of EGF, FGF-2, and TGF-β in the RRD group were higher than those in control group. Furthermore, the expression levels of EGF, FGF-2, and TGF-β in the chitosan group were higher than those in RRD group, indicating that chitosan increased the levels of EGF, FGF-2, and TGF-β in retinal tissue to accelerate the healing of retinal breaks.

Microvessel formation plays an important role in the proliferation of epithelial cells during wound healing. Vascular regeneration is also an important indicator of wound healing [37–40]. Among various substances related to the promotion of angiogenesis, VEGF plays an extremely important role. VEGF is a glycosylated secretory polypeptide that regulates the formation of new blood vessels at the molecular level, including endothelial cell proliferation, cell migration, chemotaxis, and protease [41]. Chitosan can stabilize platelet growth factors and regulate stem cell differentiation for tissue regeneration [42]. Chitosan-based functional materials promote hemostasis and anti-inflammatory effects during wound repair [43]. The results of this study showed that the expression level of VEGF in the RRD group was higher than that in the control group, and the expression level of VEGF in the chitosan group was higher than that in the RRD group, indicating that chitosan increased the content of VEGF in retinal tissue to promote wound healing.

Inflammation is a key process in wound repair [44–46]. IL-6 is produced by vascular endothelial cells and monocytes that regulate inflammatory reactions in both directions. During early trauma, appropriate IL-6 expression improves host defense ability and prevents tissue damage. However, IL-6 overexpression can mediate excessive inflammation [47,48]. IL-8 is a CXC chemokine synthesized and secreted by macrophages, vascular endothelial cells, fibroblasts, and epithelial cells. It plays an important role in the inflammatory response, immune response, and antitumor activity. IL-8 has strong chemotactic and activation effects on leukocytes. It enables neutrophils to gather, adhere, activate, and release lysosomal enzymes at inflammatory sites, while simultaneously causing T lymphocyte recycling, directional migration, and chemotaxis of basophils. IL-8 is involved in the pathophysiological processes of shock, ischemia/reperfusion injury, and other diseases [49]. Owing to its remarkable immunostimulatory activities, chitosan can stimulate the release of anti-inflammatory cytokines, chemokines, and growth factors, and hence promote and support every phase of wound healing, including hemostasis, inflammation, cell migration, proliferation, tissue repair, and cell regeneration [50]. Our study revealed that the concentrations of IL-6 and IL-8 in the RRD group were significantly higher than those in the control group, whereas the concentrations of IL-6 and IL-8 in the chitosan group were significantly lower than those in the RRD group, suggesting that chitosan can clear inflammatory cytokines and inflammatory mediators in local retinal tissues, reduce the degree of inflammation around retinal breaks, and promote the healing of retinal breaks.

Conclusions

In conclusion, we successfully demonstrated that chitosan can be used to seal retinal tears and RD without requir-

ing intraocular tamponade during surgery. Moreover, chitosan maintains high levels of growth factors and reduces inflammatory factors in the vitreous, which may reduce and delay the death of retinal cells and help restore visual function after retinal repositioning.

Availability of Data and Materials

All data generated and analysed during this study are included in this published article material. Furthermore, the full datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

QW carried out the experimental animal, histology, ELISA and was a major contributor in writing the manuscript. QW and YJ completed the experimental rhegmatogenous retinal detachment, clinical examination and data analysis. QW and YJ conceived the experiment and revised the manuscript. YJ confirmed the authenticity of all the raw data. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Research Ethics Committee of the Tong Ren Hospital affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China (Number: A2023-005-01). This study follows ARVO's statement regarding the use of animals in ophthalmology and vision studies.

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Conflict of Interest

The authors declare no conflict of interest.

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