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Published in:
Acta Biomaterialia

DOI:
10.1016/j.actbio.2010.11.013

2011

Citation for published version (APA):

Total number of authors:
7

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Evaluation of viscoelastic poly(ethylene glycol) sols as vitreous substitutes in an experimental vitrectomy model in rabbits

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For: Acta Biomaterialia.
Abstract

The aim of this study was to employ an experimental protocol for in vivo evaluation of sols of 5 wt.% poly(ethylene glycol) (PEG) in phosphate buffered saline (PBS) as artificial vitreous substitutes. A 20 gauge pars plana vitrectomy and posterior vitreous detachment were performed in the right eye of 8 pigmented rabbits. Approximately 1 mL of the viscoelastic PEG sols were then injected into the vitreous space of 6 eyes. PEG with an average molecular weight of 300,000 g mol\(^{-1}\) and 400,000 g mol\(^{-1}\) was used in 2 and 4 eyes respectively. Two eyes received balanced salt solution (BSS) and served as controls. Full-field electroretinography (ERG) and intraocular pressure (IOP, palpation) was measured pre-and post-operatively at regular intervals up to 41 days. The rabbits were sacrificed and the eyes were examined by retinal photography, gross macroscopic examination and histology. The viscoelastic sols were successfully injected and remained translucent throughout the postoperative period, with some inferior formation of precipitates. None of the eyes displayed IOP elevation postoperatively, but in 3 of the PEG sol injected eyes, transient hypotony was noted. One eye sustained a retinal detachment during surgery and another 2 in the postoperative period. ERG recordings confirmed preservation of retinal function in 3 out of 4 eyes injected with 400,000 g mol\(^{-1}\) PEG. Histological examination revealed upregulation of glial acidic fibrillary protein (GFAP) in Müller cells in PEG sol injected eyes, but normal overall morphology in eyes with attached retinas. The viscosity of the sol was not retained throughout the postoperative period, indicating the demand for polymer crosslinking to increase residence time. The results provide promising preliminary results of the use of PEG hydrogels as a vitreous substitute.
1. Introduction

Synthetic vitreous replacement is an important part of vitreoretinal surgery, for use as a tamponade in retinal detachment, diabetic retinopathy, trauma or possibly as a depot for long-term drug delivery. Currently gases, perfluorocarbon liquids or silicone oil are most predominately applied clinically to separate water from the retinal rupture to prevent subretinal fluid accumulation leading to further retinal detachment. However, these agents have several disadvantages, including cataracts and elevation of intraocular pressure (IOP), and they are unsuitable as long term vitreous substitutes [1-2]. The vitreous is a hydrogel composed of collagen and hyaluronic acid, with reported values of 98-99% water, pH 7.0-7.4, with an elastic modulus of 4.2-4.7 Pa and a loss modulus of 1.9-3.7 Pa and a refractive index of 1.3345-1.3348 [3]. Synthetic hydrogels are a promising form of vitreous substitute that could tamponade water influx through a rupture and simultaneously provide a normal vitreous biochemistry. Material requirements include optical translucence and homogeneity, atoxicity and physiological isotonicity. The material must also permit transport of salts, nutrients and water and be stable to enzymatic or hydrolytic degradation as well as being immunologically inert. In addition, it should be able to be administrated through a 20 gauge needle without inducing structural damage to the gel. Therefore, an injectable hydrogel that cross-links in situ is theoretically an ideal solution. A wide variety of synthetic polymers, in solution or as cross-linked hydrogels, have been tested experimentally and shown to satisfy a wide range of necessary properties. However, to date, no material has been successfully translated for clinical use that can meet all desired properties for an ideal vitreous substitute [4-15]. Problems have included gel fragmentation, opacification, retinotoxicity and IOP elevation. In a prior study, a polymer used in reconstructive surgery, poly(alkyl-imide) (Bio-Alcamid™), was tested as a potential substitute [16-17]. However, this material caused central retinal edema one day after surgery, accompanied by pathological electroretinography (ERG) [18]. Poly(ethylene glycol) (PEG) is a synthetic water soluble polymer that has been FDA approved for use
in a wide range of biomedical applications, including injectable hydrogels [19]. It has also been tested in formulations for intravitreal drug delivery, repair of scleral incisions and sealing retinal detachments [20-22]. In this study, we investigated the injection of viscoelastic sols of high molecular weight (> 200 kDa) PEG in phosphate buffered saline (PBS) into the vitreous bodies of rabbit eyes following vitrectomy. The molecular weights and concentration of PEG were chosen to approximate the mechanical properties of the natural vitreous. The aim of the study was to ascertain whether the PEG sols preserve normal intraocular pressure (IOP), retinal function and attachment as vitreous substitutes after experimental vitrectomy.
2. Materials and methods

2.1 Characterization of PEG sols

Sols of 5 wt.% poly(ethylene glycol) (PEG, Sigma Aldrich, St. Louis, MO) were prepared in sterile phosphate buffered saline (PBS, pH 7.4, Invitrogen, Carlsbad, CA), with molecular weights of 300 and 400 kDa. The sols are referred to as S300 and S400 respectively in subsequent sections. pH was measured using a digital pH probe (Russell RL060P, Thermo Scientific, Waltham, MA). Dynamic rheology was performed using an AR G-2 with a 40 mm diameter 2° cone and plate set-up (TA Instruments, New Castle, DE) at 10 rad s\(^{-1}\) oscillation and 5 % strain. Refractive indices were measured using a handheld Brix refractometer (PAL-1, Atago, Tokyo, Japan). Sols were sterilized by ultraviolet irradiation for 12 hours prior to surgery.

2.2 Animal selection and study protocol

The study was approved by The Regional Ethics Committee for Animal Experiments in Lund and it also conformed to the ARVO Resolution on the Use of Animals in Vision and Ophthalmic Research. Eight pigmented rabbits, aged 4 months were used in the experiment. The right eye was operated upon and injected with 0.8-1.5 mL solutions following vitrectomy (2 with S300, 4 with S400, 2 with balanced salt solution (BSS)) while the left eye served as a control. Examination including ophthalmoscopy and IOP measurement (palpation) was performed at postoperative day 6, 20 and 41. The rabbits were sacrificed at day 41, at which time the eyes were gross examined, photographed and prepared for histological examination.

2.3 Surgery

All procedures were performed by a clinically well experienced vitreoretinal surgeon. General anesthesia was provided with a combination of ketamine (35 mg/kg) and xylazine (5 mg/kg)
intramuscularly. The right eye was instilled with cyclopentolate (1%) and phenylephrine (10%) 30
minutes before surgery. Topical tetracaine (0.5%) was applied just before surgery. The conjunctiva was
incised limbbally 270° from 9 to 6 o’clock with a vertical incision at 12 o’clock, creating two flaps. A
20G infusion cannula was sutured to the sclera in the 4 o’clock position 1 mm posterior to the limbus
and a balanced salt solution (BSS, Endosol, Allergan Medical Optics) was started. Two 20G
sclerotomies were made in the 10 and 2 o’clock positions. A BIOM 90-D len (Oculus) was used to
visualize the fundus, and an Accurus surgical system machine (Alcon, Fort Worth, TX) was used for
surgery. A standard endo-illuminating light probe (Alcon) was introduced through the 10 o’clock
sclerotomy (illumination level 80%), and a vitreous cutter (Innovit, Alcon) was inserted through the 2
o’clock sclerotomy. Posterior vitreous detachment (PVD) was created by positioning the vitrectomy
probe at the margin of the disk and applying suction (100 mmHg) while pulling on the probe. PVD was
confirmed visually as the posterior vitreous cortex separated from the posterior pole. All vitreous in the
central fundus (approximately 50% of the total volume) was removed while peripheral parts were left
because of the risk of instrument touch to the comparatively large lens. In one case (#1 S300) a retinal
touch with the vitrector occurred causing a retinal rupture and anterior chamber bleeding. After
vitreous removal, a fluid-air exchange was performed after which PEG sol was injected under visual
control through a 19 gauge needle. The amount of sol injected varied from 0.8 to 1.5 mL (Table 2). In
one case, some backflow during gel injection was noted (#6 S400). The sclerotomies and conjunctiva
were sutured and 25 mg gentamicin and 2 mg betamethasone were injected subconjunctivally. No
postoperative treatment was given. The eyes were examined externally daily and with an external
ophthalmoscope at days 6 and 20. Images of the retinas were taken at day 20 and day 41 (RetCam,
Clarity Medical Systems, Pleasanton, CA). Two eyes, used as controls, underwent the above surgery
but were injected with balanced salt solution (BSS) instead of PEG sol.
2.4 Full-Field ERG

A standardized full-field electroretinography (ERG) was recorded 7 days before surgery and 41 days postoperatively on the right eye using a Nicolet Viking analysis system (Nicolet Biomedical Instruments, Madison Wisconsin) as previously described [23]. During examination the rabbits were sedated with Hypnorm, (fentanyl 0.2 mg/ml and fluanisone 10 mg/ml) 0.1 ml/kg, intramuscularly and the pupils were dilated with Cyclogyl (cyclopentolate hydrochloride 1%) to a pupil diameter of 8–9 mm. After 30 minutes of dark adaptation a Burian-Allen bipolar ERG contact lens electrode was applied on the topically anesthetized cornea together with a subcutaneous ground electrode on the neck. The lens was lubricated with methylcellulose (2%). Responses were obtained with a wide band filter (-3 dB at 1 Hz and 500 Hz), stimulating with single full-field flashes (30 μs) with dim blue light (Wratten filters # 47, 47A and 47B) and of white light (0.8 cd.s/m2). Cone responses were obtained with 30 Hz flickering white light (0.8 cd s/m2) averaged from 20 sweeps. The luminances of the three different light stimuli refer to the light reflected from the Ganzfeld sphere.

2.5 Tissue preparation

At day 41, the rabbits were sacrificed and the eyes were dissected, gross examined and fixed for 1 h in 4% formalin, pH 7.3 in a 0.1 M Sørensen’s phosphate buffer (PB). After fixation, the specimens were washed with 0.1 M Sørensen’s PB, and then washed again using the same solution containing sucrose of rising concentrations (5-25%). The specimens were sectioned at 12 μm on a cryostat, and each 10th slide was stained with hematoxylin and eosin according to standard procedures. To explore Müller cell activation, glial fibrillary acidic protein (GFAP) immunolabeling was performed by washing sections from each eye in 0.1 M of sodium phosphate-buffered saline pH 7.2 (PBS) with 0.25% Triton X-100 (PBS/Triton) and incubating them with the primary antibody (anti-GFAP, clone G-A-5; Boehringer Mannheim Scandinavia, Bromma, Sweden, diluted 1:4 with PBS/Triton with 1% bovine serum albumin) overnight at +4°C. After incubation, the slides were rinsed in PBS/Triton, incubated with
fluorescein isothiocyanate (FITC)-conjugated antibodies for 45 min, rinsed, and mounted in custom-made anti-fading mounting media. Unoperated eyes served as controls. For negative controls, the same labeling procedure without the primary antibody was performed on both the normal left and the operated right eye of the animals.
3. Results

3.1 Sol physical properties

Table 1 shows the physical properties measured for the sols. The addition of PEG to pH 7.4 PBS resulted in a decrease in pH, which remained above 7 in all cases. Both the elastic and loss moduli increased with increasing molecular weight at constant PEG concentration in PBS of 5 wt.%.

Refractive indices decreased slightly with increasing molecular weight and were approximately nD of 1.339 at 25ºC converted from Brix % in all sols. While the sols permitted transmission of visual information, the sols were not as clear as pure water (Fig. 1A). Complete transparency of a vitreous substitute is required for clinical use. Transparency appeared unchanged when heating the sols from 4 ºC to 37 ºC. S300 and S400 could be drawn into a syringe via a 19 gauge cannula and this appeared unchanged at 37 ºC. A small amount of PEG remained insoluble, observed by addition of a blue hydrophilic dye to the sols. This fraction was larger in S400 compared with S300 (Fig. 1B).

3.2 Post-operative evaluation

Results are summarized in Table 2. During surgery, one eye had a retinal rupture and bleeding in the anterior chamber due to contact with a surgical instrument (#1 S300). In all other eyes the PEG sols were injected without complications. Six days post-operatively, two eyes injected with the PEG solutions displayed hypotony, with an intra-ocular pressure (IOP) approximately 0 mmHg (#1 S300, #8 S400), while the remaining 4 had an IOP of approximately 10-15 mmHg. Hypotonic eyes had a slight cataract superiorly but the majority of the lens was clear. No signs of inflammation were observed. The eye with a per-operative retinal rupture displayed a retinal detachment of approximately 40% (#1 S300), while remaining eyes displayed a normal retina. The vitreous space was clear in the central and superior part. However, all eyes containing PEG displayed small white aggregates in the inferior
vitreous body (Fig. 2). Twenty days post-operatively, this precipitate was still present, but appeared to be reduced in quantity. At this time, one eye had a total retinal detachment (#1 S300) (Fig. 3 A). Two eyes had peripheral retinal detachments (#2 S400, #6 S400). No inflammation was observed externally or internally (Fig. 3 C, E). A slight cataract was noted in two of the PEG injected eyes (#2 S400 and #5 S300), and 1 BSS eye. IOP was not elevated in any eye but 2 eyes with detachment were hypotonic, between 0-5 mmHg (#1 S300, #6 S400). At final examination and dissection, 41 days post-operatively, the vitreous was clear in all animals (Fig. 3 B, D, F), and the IOP was normal in all eyes. Minimal cataract was noted in three eyes. Precipitates were found in the inferior vitreous in 2 eyes (#6 S400, #8 S400), but was reduced compared to earlier examination time points. The retina was completely detached in two eyes (#1 S300, #6 S400) and some folding was also noted (#4 BSS, #5 S300, #6 S400) (Fig. 3 F). The sols in all eyes were completely resorbed.

### 3.3 Histology

In hematoxylin and eosin stained sections, 3 of the PEG sol injected eyes, and the 2 BSS injected eyes displayed a normal retinal morphology (Fig. 4A, B; Table 2). The eye with an early clinically diagnosed retinal detachment (#1 S300) displayed destruction of the retinal layers, choroidal inflammation and dissolved retinal pigment epithelium (RPE) (Fig. 4C). In another eye with detachment (#6 S400), outer and inner photoreceptor segments were missing and some cell loss in all nuclear layers was evident. In one eye (#5 S300), a minimal central detachment was found together with inflammatory cells in the outer nuclear layer (ONL) and subretinal space (Fig. 4D). The macroscopically observed precipitates seen in #2 S400 were observed in histological sections as brown clumps on the retinal surface, and also in an area within the inner retinal layers, with presence of macrophages (Fig. 4 E, F).
3.3.1 Immunofluorescence labeling

In BSS injected eyes GFAP labeling was comparable with normal unoperated controls with discrete labeling of Müller cells seen only in the periphery (Fig. 5 A). All eyes injected with PEG sols displayed upregulation of glial fibrillary acidic protein (GFAP), as a sign of Müller cell activation (Fig. 5 B). The eye with surgically induced total retinal detachment displayed disorganized Müller cells (#1 S300) whereas in all other eyes, Müller cells displayed the normal vertical arrangement.

3.4 Electretinography

Full-field electretinography (ERG) measurements were obtained pre- and post-operatively in all 8 eyes (Table 3). All but one case followed the histological results. No residual response could be measured in the two eyes with total retinal detachment (#1 S300, #6 S400). Another eye, with a minimal central detachment, also displayed no response (#5 S300). Two eyes displayed supernormal responses to blue light (#4 BSS) and blue and white light (#7 S400). The other eyes did not display marked changes relative to pre-operative baseline recordings (#2 S400, #8 S400, #3 BSS).
4. Discussion

The vitreous is a hydrogel composed of collagen and hyaluronic acid, with reported values of 98-99% water. pH 7.0-7.4, with an elastic modulus of 4.2-4.7 Pa and a loss modulus of 1.9-3.7 Pa and a refractive index of 1.3345-1.3348 [3]. 5 wt.% PEG sols with average molecular weights between 200,000 and 400,000 g mol\(^{-1}\) had viscoelastic properties with the same order of magnitude as the natural vitreous. However, none of the individual materials completely matched both the elastic and loss moduli of the natural vitreous. Using phosphate buffered saline (PBS), the pH values of the resulting sols were slightly above 7, and both sols had similar refractive indices (1.339 at 25ºC) to the natural vitreous. PEG sols presented an attractive vitreous substitute to test in an experimental vitrectomy model, especially given promising results indicating ocular biocompatibility of PEG hydrogels [20-22].

The sols formed some small white aggregates in the inferior vitreous body in vivo, resulting in a lack of total transparency, which is not ideal for clinical use. Both eyes injected with S300 had retinal detachment and loss of electrical function (#1, #5). In one eye, the retinal detachment was caused during surgery (#1). In the other, a minimal central detachment and subretinal ONL inflammation developed in the postoperative period as observed upon histological examination (#5). It is uncertain why this eye had no postoperative ERG responses after 41 days, but inflammation may be partly responsible. The tolerability of S300 was inconclusive, limited by the small sample size. In 3 out of 4 eyes with S400, the PEG sol had a minimal effect on retinal morphology and electrical function (#2, #7, #8). One eye with S400 developed a postoperative retinal detachment (#6). However, all eyes injected with PEG sols exhibited elevated GFAP expression, indicative of retinal stress and Müller cell activation, which was not observed in BSS eyes. S400 affected the retina to a lesser degree than a previously tested material, poly(acryl-imide), which caused retinal edema [18].
Following removal of the natural vitreous, metabolic and physiological changes occur within the eye, involving oxygen concentration, osmotic balance and molecular transport that may affect both the lens and the retina [24, 25]. The normal morphology and non-pathological ERG responses observed in 3 out of 4 eyes injected with S400 may indicate that the nutritional demands of the retina are met by the PEG sol. The supernormal rod ERG (blue light) responses observed in one BSS eye (#4) and one S400 eye (#7) may potentially be related to protein kinase C (PKC) alpha downregulation and GFAP upregulation and warrants further investigation [26]. IOP was measured by digital palpation, which is not the most accurate and reliable method for IOP measurement. However, we were primarily interested in detecting any elevation in IOP, for which palpation was adequate. The PEG sols did not result in IOP elevation, an important clinical consideration. One eye was hypotonic post-operatively (#8 S400) and returned to normal within 20 days. Hypotonia in the first week following vitreoretinal surgery is not uncommon, and usually related to mild inflammation of the ciliary body. The rabbits were not treated with anti-inflammatory eye drops as is common practice in the clinic which may explains this phenomenon. Our protocol for vitrectomy in the rabbit eye is well established, and from experience IOP returns to normal within 1-2 weeks. The PEG sols were not retained in the vitreous body throughout the post-operative period. In retinal detachment surgery 3 to 7 days are required for laserpexy to form a permanent adhesion between the retina and choroid/RPE and complex cases of proliferative vitreoretinopathy (PVR) may require a tamponade for several months [27]. Despite a small sample size, the data provides evidence that in their current form PEG sols are not ideal due to their interaction with the retina and limited retention time. Steps should be taken to minimize interaction with the retina. Cross-linking of the polymer may improve biocompatibility and retention time as well as mechanical properties [28]. Future work will focus on cross-linking PEG following injection, so that the resulting viscoelastic insoluble gel is retained for a longer period in the vitreous body. This could be achieved, for example, through the use of PEG with thiol and acrylate functional groups to form cross-links in situ [29, 30].
5. Conclusion

In the present study, aqueous sols of poly(ethylene glycol) (PEG) and phosphate buffered saline (PBS) were tested as potential vitreous substitutes in a rabbit model. Sols of 5 wt.% PEG with a molecular weight of 400,000 g mol\(^{-1}\) in PBS were shown to have mechanical and optical properties similar to the natural vitreous and were well tolerated by the retina with minimal histological or electrophysiological changes, with the exception GFAP upregulation over a period of 41 days. However, the sols were not retained in the posterior body throughout the postoperative period. These results indicate the use of a cross-linked PEG hydrogel as a potential artificial vitreous substitute.
6. Acknowledgments

This study was supported by The Faculty of Medicine, University of Lund, The Swedish Research Council, The Torsten and Ragnar Söderberg Foundation, and research funds of Departments of Ophthalmology Örebro. C.D.P. was supported by the MIT/CIMIT Medical Engineering Fellowship and a gift to MIT by InVivo Therapeutics Corporation.
References


Figure captions

Figure 1. Poly(ethylene glycol) (PEG) sols. A. Transparency of PEG sol (left) compared to distilled water (right). B. Addition of hydrophilic blue dye to PEG sols showed a hydrophobic precipitate. The hydrophobic region was larger, the higher the molecular weight of PEG. S400 (left), S300 (right).

Figure 2. Ophthalmoscopic examination 6 days postoperatively. PEG injected eyes displayed white precipitate in the inferior vitreous body. DPO = days postoperatively.

Figure 3. Images of retinas 20 days postoperatively (A, C, E) and eyes at dissection 41 days postoperatively (B, D, F). A-B. #1 S300. Total retinal detachment caused during surgery (A) with no sol remaining (B). C-D. #7 S400. Eye injected with PEG sol was normal with retina attached (C) and no sol remaining (D). E-F. #4 BSS. Eye injected with balanced saline solution displayed attached retina (E) and minimal retinal folds (F). DPO = days postoperatively.

Figure 4. Hematoxylin and eosin (H&E) stained sections. A-B. #4 BSS (A) and #7 S400 (B) displayed normal retinal morphology. C. #1S300. Total destruction of the retina with choroidal inflammation and dissolved retinal pigment epithelium (RPE). D. #5 S300. The retina had some elongation of Müller cell nuclei, large cells in the ONL (black arrow) and subretinal inflammatory cells (white arrows). E-F. #2 S400. Brown precipitate and macrophage invasion on retinal surface (E) and inner retinal layers (F) (white arrows). Scale bar = 400 microns (C), 100 microns (E), 50 microns (A, B, D, F). DPO = days postoperatively, VITR = vitreous up, SCL = sclera down, NFL = neurofilament layer, GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, IS = photoreceptor inner segments, OS = photoreceptor outer segments, RPE = retinal pigment epithelium.

Figure 5. Glial fibrillary acidic protein (GFAP) immunofluorescence stained sections of the peripheral retina. A. #3 BSS. Eye injected with balanced saline solution shows labelling of the normal arrangement of vertically arranged Müller cells. B. #2 S400. Eye injected with PEG sol S400 displayed upregulation of GFAP in Müller cells. Scale bar = 50 microns. DPO = days postoperatively.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Table 1.

<table>
<thead>
<tr>
<th>Sol name</th>
<th>PEG molecular weight (kDa)</th>
<th>pH</th>
<th>Elastic modulus G’ (Pa)</th>
<th>Loss modulus G” (Pa)</th>
<th>Refractive index at 25 ºC (Brix %)</th>
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Sol physical properties
### Table 2.

<table>
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<th>Case # and vitreous substitute</th>
<th>Injection volume, Surgical notes</th>
<th>Day 6</th>
<th>Day 20</th>
<th>Day 41</th>
<th>Dissection</th>
<th>H&amp;E</th>
<th>GFAP</th>
</tr>
</thead>
</table>
| **#1 S300**                  | 0.8 mL  
| **#2 S400**                  | 0.9 mL  
Minimal conj. injection.  
| **#4 BSS**                   | Not examined | No injection. Cornea clear. Mild cataract corresp. to the right sclerotomy. Vitreous clear IOP 10 (palp). | Clear, retina ok, IOP ok | Minimal retinal folds, artefact? | Normal | Minimal upregulation |
| **#5 S300**                  | 1.3 mL  
Minimal injection. Precipitate in the vitreous inferiorly. Minimal cataract. IOP approx. 10 | No injection. Cornea clear. Mild cataract. Vitreous clear. Some precipitate inferiorly. Retina ok. IOP 10 (palp). | Minimal cataract, vitreous clear without precipitate. Retina ok, IOP ok | Some retinal folding. No gel | Minimal central detachment (fold?). Retina looks ok, but inflammatory cells subretinally and large cells in the ONL are present on some sections | Some elongation of Müller cell nuclei |
| **#6 S400**                  | 1.5 mL  
(some backflow)  
| **#7 S400**                  | 1.2 mL  
Minimal injection. Precipitate in the vitreous inferiorly  

Pre-, per-, and post-operative data. H&E = hematoxylin and eosin stained histologic sections. GFAP = glial fibrillary acidic protein immunofluorescence labeled sections.
Table 3.

<table>
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<tr>
<th>Eye # and vitreous substitute</th>
<th>Blue light</th>
<th>White light</th>
<th>30 Hz flicker</th>
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<tr>
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<tr>
<td>#8 S400</td>
<td>56.0</td>
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<td>65.8</td>
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</table>

Pre- and post-operative (day 41) ERG data. Values are given of the wave amplitudes (µV) for rod ERG (blue light), combined ERG (white light), and dark-adapted single-flash cone ERG (30 Hz flicker).