A Self-Assembling Peptide Gel as a Vitreous Substitute: A Rabbit Study

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PURPOSE. To evaluate a self-assembling peptide gel as a potential vitreous substitute.

METHODS. PanaceaGel SPG-178, a self-assembling peptide gel, was diluted with distilled water and a balanced salt solution to achieve a final peptide concentration of 0.1%. The gel’s refractive index, visible light transmission rate, and rheologic properties were investigated. The gel’s biocompatibility was evaluated by examining the cellular viability (live and dead staining) and proliferation rate (alamarBlue assay). A 25-G pars plana vitrectomy was performed on the right eye of 21 New Zealand white rabbits. The gel was then injected into the vitreous cavity of 15 eyes. Six eyes were injected with a balanced salt solution (BSS) and served as controls. Toxicity was examined using electroretinography and histologic analysis after the injection of the gel.

RESULTS. The gel’s physical properties closely resembled those of human vitreous. The gel showed no apparent toxicity. When the gel was injected into the vitreous cavity, fragmentation was not observed. Additionally, the gel remained transparent in the vitreous cavity and no complications were observed for 3 months after the injection. Electroretinography and histology confirmed the gel’s biocompatibility.

CONCLUSIONS. This diluted self-assembling peptide gel could be provide a promising vitreous substitute.

Keywords: vitreous humor, retina, self-assembling peptide gel

The vitreous humor is a gel-like substance that fills the vitreous cavity of the eye. It is transparent and plays a crucial role in maintaining the shape of the eye and supporting the retina. The vitreous humor is composed of a complex mixture of proteins, carbohydrates, and water. However, vitreous degeneration is a common pathological condition that occurs with age, leading to complications such as retinal detachment and glaucoma. Therefore, the development of a safe and effective vitreous substitute is essential.

Currently, there are several vitreous substitutes available, such as silicone, gas, and hydrogel. Silicone is commonly used as a vitreous substitute due to its low toxicity and good biocompatibility. However, silicone can lead to complications such as inflammation and migration into the retina. Gas, such as air and expandable gas, is used as a short-term substitute, but it is not suitable for long-term use due to its low refractive index and absorption. Hydrogels, on the other hand, have the potential to be used as long-term substitutes due to their adjustable properties and biocompatibility.

A self-assembling peptide, SPG-178, spontaneously assembles itself into nanofibers, creating a stable β-sheet structure in water. These nanofibers can be used as a functional substitute for vitreous due to their adjustable properties and biocompatibility.

In this study, we evaluated a self-assembling peptide gel as a potential vitreous substitute. The gel’s properties and biocompatibility were examined in rabbits for 3 months after injection of the substitute following pars plana vitrectomy (PPV).
METHODS

Preparation of the Vitreous Substitute

Powdered 1% (wt/vol) PanaceaGel SPG-178 (CH$_3$CO-2RLDLRLALRLDLR-NH$_2$; [R, L, D and A stand for arginine, leucine, aspartic acid, and alanine, respectively]) was diluted in distilled water to achieve a 0.15% (wt/vol) gel with a pH between 6.0 and 7.5. The resulting solution was further diluted with OPEAQUA, a balanced salt solution, (Kowa, Tokyo, Japan) to achieve a 0.1% (wt/vol) gel. The 0.1% (wt/vol) gel was then packaged aseptically in a 5-mL syringe and attached to a 27-G needle (Fig. 1b).

Physical Property Measurement

A refractometer (ATAGO Refractometer NAR-1T; Abbe, Kirkland, WA, USA) was used to measure the refractive index of the vitreous substitute at 37°C. The test performed using visible light at a wavelength of 552 nm. The gel’s visible light transmission was measured using a spectrophotometer (UV-3150; SHIMADZU, Kyoto, Japan) at room temperature. The gel’s viscoelastic properties were evaluated with a rheometer (AR-1000; TA Instruments, New Castle, DE, USA); testing was performed at 37°C with 3% strain. The testing frequency was varied between 10 and 0.1 rad/s.

Biocompatibility

Cell Viability Assay. ARPE19 cells (CRL-2302; American Type Culture Collection [ATCC], Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and F12 medium (ATCC) containing 10% fetal bovine serum. After overnight culturing, the medium was replaced with the vitreous substitute and fresh medium (Fig. 3a). Cells were again cultured overnight. The gel and the medium were removed and fresh medium containing calcein acetomethoxy (AM) 1 µg/mL and propidium iodide (PI) 2.7 µg/mL (Dojindo, Kumamoto, Japan) was added to stain live and dead cells, respectively. After 2 hours of incubation at 37°C, the stained cells were observed by fluorescence microscopy. Negative controls (cells without the gel) and positive controls (cells treated with 0.3% of Triton X-100 in PBS; Invitrogen, Carlsbad, CA, USA) were also set and examined.

Cell Proliferation Assay. After the overnight ARPE19 cell culture, the medium was removed and gel and fresh medium were added to the cells (Fig. 3a). A control culture without the substitution was also set. After an additional 72 hours of culture, all media were replaced with fresh medium consisting of 10% alamarBlue (Invitrogen, Carlsbad, CA, USA). After a 2-hour incubation, the medium was collected and fluorescence (excitation wavelength = 560 nm, emission wavelength = 590 nm) was measured.

Animal Preparation

Twenty-one female New Zealand white rabbits weighing 2.0 to 2.5 kg were used in this study. Animals were cared for and handled in accordance with the ARVO Statement for the Use of Animals in Vision and Ophthalmic Research and in accordance with the institutionally approved protocols of the Animal Experiment Committee of Osaka University. Rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide (Midrin P; Santen, Osaka, Japan) and phenylephrine (2.5%).

Vitrectomy and Injection of the Vitreous Substitute

A 25-G sutureless trocar-cannula vitrectomy system, provided by Alcon Laboratories (Fort Worth, TX, USA), and an Ocutome (Alcon) were used to perform PPV on the right eye of each rabbit. The 25-G trocar-cannula was inserted 2.5 mm from the limbus. Under the microscope, a core vitrectomy was performed and a posterior vitreous detachment was created. The detachment was then expanded to remove as much of the posterior vitreous as possible. A gentle peripheral vitrectomy, paying close attention to avoid the lens, was performed. This minimized the likelihood of a postoperative cataract formation.

Fifteen rabbits underwent PPV and replacement with the 0.1% (wt/vol) PanaceaGel peptide hydrogel. Six rabbits underwent PPV and replacement with a balanced salt solution (BSS plus; Alcon), and served as controls. The incision sites were closed without sutures. In the vitreous substitute group, four eyes were harvested at postoperative day 3, four eyes were harvested at postoperative day 7, and two eyes were harvested at postoperative day 28 (1 month) and five eyes were harvested at postoperative day 84 (3 months). In the control group, one eye was harvested at postoperative day 3, one eye was harvested at postoperative day 7, and two eyes were harvested at day 28 (1 month) and two eyes were harvested at postoperative day 84 (3 months).
Pre- and Postoperative Examinations

Slit-lamp biomicroscopy (Topcon Corp., Tokyo, Japan) and fundoscopy (Topcon Corp.) were performed on the right eye preoperatively and postoperatively on days 7, 14, 21, 28, 56, and 84. The IOP was also measured on the right eye preoperatively and postoperatively on days 7, 14, 21, 28, 56, and 84 using a tonometer (TONOLAB; Icare Finland Oy, Helsinki, Finland).

Electroretinography

Electroretinography (ERG) was conducted preoperatively and on postoperative days 7, 14, 21, 28, 56, and 84 in the right eyes of all rabbits using the Neuropack system (Nihon Kohden Corp., Tokyo, Japan). All recordings were taken after the right pupil was dilated (0.5% tropicamide, Midrin P; Santen) and sufficient dark adaptation. After application of 0.4% oxybuprocaine (Benoxil; Santen), a contact lens electrode lubricated with 1.5% hydroxyethyl cellulose (Scopisol; Senju, Osaka, Japan) was applied to the cornea. A grounding needle electrode was also inserted into the skin of the forehead.

Histopathology

The rabbits were euthanized and their eyes were immediately enucleated for tissue preparation. The anterior segments were dissected, and the posterior eyecups were fixed in 4% paraformaldehyde solution and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Data Analyses

Data are presented as the mean ± SD. ANOVA was used to determine the difference between the IOP measured preoperatively and that measured at each time point postoperatively by JMP software (SAS Institute Inc., Cary, NC, USA). The Student’s t-test was used to determine the statistical significance for the proliferation test. Statistical significance was defined as P less than 0.05.
RESULTS

Physical Properties

The refractive index of the vitreous substitute was $1.3339 \pm 0.0001$ and the visible light transmission rate was $96.7 \pm 0.7\%$. The substitute had a storage modulus ($G'$) that was higher than the loss modulus ($G''$) at all frequencies. This indicated that the solution exhibited a typical "gel-like" behavior. It had a $G'$ of $18.12 \pm 2.00$ Pa at 1 rad/s (Fig. 2).

Biocompatibility

There was no difference in the fluorescence staining between the retinal pigment epithelial (RPE) cells incubated in the vitreous substitute for 24 hours (Fig. 3b) and the negative controls (Fig. 3c). In contrast, nearly all RPE cells incubated with Triton X-100 exhibited a red fluorescence stain (Fig. 3d). The proliferation of RPE cells in the vitreous substitute group was not significantly different from that in the control group ($P > 0.05$; Fig. 3e).

Pre- and Postoperative Examinations

Slit-lamp examination revealed no significant inflammation, hemorrhage, or other disease on postoperative days 1, 3, 7, 14, 21, 28, 56 (2 months), and 84 (5 months). Additionally, no rabbits developed cataracts during the follow-up period (Fig. 4a). Fundoscopic examination also revealed no abnormal findings in the optic nerve or ocular fundus (Fig. 4b). The vitreous substitute remained transparent, with no opacities observed. The IOP remained within the normal range and was not significantly different between the control and vitreous substitute groups at any time point examined (Fig. 5).

FIGURE 5. Intraocular pressure in the rabbits before and after PPV. No IOP elevations were observed during the follow-up period. The IOP was not significantly different between the control and vitreous substitute groups at any time point examined (ANOVA, $P > 0.05$).
Electroretinography

No significant abnormalities in both the control and vitreous substitute groups were observed on the electroretinograms. The a- and b-wave amplitudes were comparable between groups and remained within the normal range during the entire follow-up period (Figs. 6a–d). The postoperative implicit times were not significantly different from the preoperative times in either group at any time point examined (Fig. 6e).

Histopathology

Histopathologic examination of the excised retinal tissue showed no degeneration, inflammation, or structural changes.
DISCUSSION

The ideal vitreous substitute would be stable, biocompatible, and have physical properties similar to that of the human vitreous. It would also have a low risk for complications and have the ability to be injected through a smaller gauge needle without fragmentation. Gel fragmentation can result in vitreous opacities, which can result in decreased vision. Given that a 27-G vitrectomy system has been developed, this characteristic is particularly important.

The physical properties of our vitreous substitute were similar to those observed for human vitreous. This is particularly important for maintaining the refractive properties of the eye, which can heavily influence visual recovery following surgery. In fact, silicone oils, which are widely used as vitreous substitutes in clinical practice, have a refractive index of approximately 1.4 and induce a hyperopic shift after surgery. Silicone oils also have other disadvantages, including increased postoperative cataract formation and glaucoma development and the need for surgical removal. Additionally, silicone oil is hydrophobic, so it is not possible to completely fill the vitreous cavity with it. As a result, a fluidic space is created where growth factors can accumulate and proliferative membranes can form. The same problem also occurs when other hydrophobic vitreous replacements (e.g., air and expansive gas) are used. However, our vitreous substitute is a hydrophilic hydrogel, which can completely fill the vitreous cavity.

Our vitreous substitute primarily consisted of PanaceaGel, which was recently verified to be a biocompatible material. These biocompatibility studies tested higher concentrations of the SPG-176 peptide than was used to create our vitreous substitute. Therefore, our vitreous gel is very likely biocompatible as well. In support of a good safety profile, cell viability and proliferation, and the results of the pre- and postoperative retinal findings observed in both the control and vitreous substitute groups (Figs. 7e–h) on postoperative days 3 (Figs. 7a, 7e), day 7 (Figs. 7b, 7f), day 28 (1 month; Figs. 7c, 7g), or day 84 (3 months; Figs. 7d, 7h).

FIGURE 7. Histologic retinal findings on postoperative days 3 (a, e), day 7 (b, f), month 1 (c, g), and month 3 (d, h). No differences were observed between eyes injected with the vitreous substitute (e–h) and those injected with the balanced salt solution (a–d).
A Self-Assembling Peptide Gel as a Vitreous Substitute

FIGURE 8. A schematic diagram of the self-assembling peptide vitreous substitute. Shear stress created during injection resulted in a sol-state, but the polymer rapidly reassembled itself into a gel once in the vitreous cavity.

postoperative examinations were not significantly different between the vitreous substitute and control groups in this study. The IOP was not significantly different before and after PPV and vitreous replacement in either the vitreous substitute or control groups. However, on postoperative day 3, the IOP was lower than the preoperative values in both study groups. The change in IOP was more pronounced in the control group, which likely experienced more aqueous humor leakage out of the incision site. We speculate that the more viscous, hydrophilic vitreous substitute completely filled the vitreous cavity, thereby sealing the incision site from inside of the eye.

Our vitreous substitute also has the advantage of self-assembly. When physical forces break down the hydrogel, the gel becomes a sol, but spontaneously re-assembles and returns to a gel state. It should also be noted that the physiological environment accelerates this self-assembly process.53,54 This is important because shear stresses induced by injecting the gel through a small-gauge instrument, could cause the gel to break. However, our vitreous substitute rapidly returned to its gel state once in the vitreous cavity (Fig. 8). Based on the slit-lamp biomicroscopy and fundoscopy findings, we demonstrated that our vitreous substitute did not fragment following injection through a 27-G needle.

In this study, the in vivo data did not indicate an overt toxic effect in the rabbit eyes over the 3-month follow-up period. Future studies with longer follow-up periods are needed to better understand the long-term safety and efficacy of our vitreous substitute. Additionally, we were not able to verify the tamponade property of the vitreous substitute. With regard to this point, before application in human subjects, it is necessary to evaluate the properties and safety of the gel by using retinal detachment models in primates. In our research, we did not analyze the influence of the substitute on various examinations, such as ultrasound and optical coherence tomography examinations. We performed various ophthalmic examinations on the eyes that received the injection, but we need to observe the effects using other examinations as well. Moreover, we did not confirm whether the substitute could be removed from eye if re-detachment occurred or if removal for other reasons was necessary. However, we confirmed that the substitute could be removed in vitro, using a typical 25-G vitreous cutter system (Supplementary Material). Henceforth, it is necessary to confirm the ability to remove the vitreous substitute in vivo as well, and to investigate safety before removal. Nevertheless, our novel vitreous substitute is promising in that it meets the requirements of a vitreous substitute; indicating its potential for use in future, smaller-gauge surgical systems.

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