Experimental Vitreous Substitution

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Introduction

Surgical removal of the vitreous is often necessary to preserve the vision of patients suffering from vision threatening conditions such as retinal detachment, penetrating ocular trauma, proliferative diabetes retinopathy, and macular holes. Vitrectomy is a surgical intervention wherein the vitreous is cut and aspirated during an infusion of Balanced Salt Solution (BSS). Expanding gases or silicone oil are often used to replace the removed vitreous providing a tamponading effect. The current compounds in use for vitreous replacement are known to cause side-effects and discomfort such as inflammation, retinal toxicity, raised intraocular pressure, and low vision during the healing period. Additionally, there is often a need for prolonged prone positioning of the head or further surgical treatment to remove the tamponade, leading to longer periods of convalescence.

The human vitreous is a gel predominantly composed of water, which fills the inner part of the posterior segment of the eye. Its purpose is not fully known, but it is believed to be of importance during embryogenesis, serve as structural support, and have a passive metabolic role. The vitreous can be seen as a hyaluronic acid hydrogel reinforced by a network of collagen. It consists of 98-99% water, and has a pH of 7.0-7.4. The structure is non-homogenous with an increase of hyaluronic acid posteriorly corresponding to a higher viscosity. The vitreous allows for diffusion of nutrients and other molecules and affects the solute transportation. One example includes the existing gradient of oxygen from the retina to the posterior surface of the lens, which has been shown to be affected by vitrectomy. With increasing age, the vitreous liquefies, a process which is associated with posterior vitreous detachment, and several pathological conditions.

The research into vitreous substitutes predates modern ophthalmic surgery. However, the seemingly simple task of devising a substitute for the relatively inert vitreous has become more and more complex as increased knowledge is gained of its role in the physiology and pathophysiology of the eye. The desired properties, along with the clinical demands of practical usability and function make up a long list, all of which are not easily achieved in one single compound. The ideal substitute should have a refractive index and physiological properties that closely mirror those of the normal vitreous, it should be retained in the eye for an extended time, and should not affect the tissues or normal physiology of the eye negatively. Additionally, there must be a feasible way to transfer the substitute into the eye, while retaining these properties. This has proven to be a daunting task, which is clearly shown by the plethora of different compounds involved in past and current research.

Aim

This project aims to create a synthetic polymer gel with suitable mechanical and physiological properties for use as an experimental vitreous substitute, with the ultimate goal of clinical use. Future possibilities include pharmacological slow-release drug delivery to the retina.
Papers and current projects


In this study, a new model for in vitro assessment of vitreous substitute candidates was evaluated. The biological impact of three vitreous substitute candidates on explanted adult rat retina was explored in a retinal explant culture model for up to 10 days. The substances evaluated, a polyalkylimide hydrogel (Bio-Alcamid®), a two component polyethylene glycol based hydrogel (PEG), and a cross-linked sodium hyaluronic acid hydrogel (Healaflow®), were either previously studied by our group in an in vivo rabbit vitrectomy model (Bio-Alcamid® and PEG) or otherwise deemed to be promising candidates for vitreous substitution (Healaflow®). Gel-exposed explants were compared with explants incubated under standard tissue culture conditions.

The explants kept under standard conditions as well as PEG-exposed explants displayed disruption of retinal layers with increased pyknosis and TUNEL labeling, signs indicative of cell death. Bio-Alcamid®-exposed explants displayed severe thinning and disruption of retinal layers with massive cell death. Healaflow®-treated explants displayed normal retinal lamination with better preservation of the retinal architecture compared with control specimens, and almost no signs of apoptosis.

The novel in vitro model provides a method of biocompatibility testing prior to more costly and cumbersome in vivo experiments. The explant culture system imposes reactions within the retina including disruption of layers, cell death and gliosis, and the progression of these reactions can be used for comparison of vitreous substitute candidates. In summary, Bio-Alcamid® had strong adverse effects on the retina which is consistent with results of prior in vivo trials. PEG gel elicits reactions similar to the control retinas whereas Healaflow® shows protection from culture-induced trauma indicating favorable biocompatibility and was thus deemed as a candidate for further trials.


Healaflow®, the new vitreous substitute candidate investigated above, was further explored in an in vivo rabbit vitrectomy model to further elucidate its potential. The experimental platform used in the groups prior studies with PEG and Bio-Alcamid® was adapted to an updated protocol with combined 25- and 20-gauge pars plana vitrectomy. After vitrectomy under general anesthesia, approximately 1 ml Healaflow® was injected into the vitreous space. Clinical evaluation, measurement of intraocular pressure (IOP), and full-field ERG were performed postoperatively. The rabbits were sacrificed at different time points between 42 and 105 days. After enucleation, the eyes were examined macroscopically, photographed, and prepared for histological examination with routine microscopy and immunohistochemistry. The unoperated eyes were used as controls.

Healaflow® was not seen to affect retinal morphology or function negatively during long-term use as a vitreous substitute, making it highly interesting in this setting. An estimated retention time of a few weeks suggests potential for use as a short term tamponade. Future work will include an increased ratio of cross-linking to prolong the structural integrity of the gel.
3) **Cellular CD68– and CD45r0.positive inflammatory responses of vitrectomy with vitreous substitutes vitrectomy with vitreous substitutes.** Henrik Barth, Sven Crafoord, Karin Arnér, Fredrik Ghosh. Poster at ARVO 2016. To be expanded into an article for publication in 2016/2017, title to be changed.

A key limiting factor in the development of vitreous substitutes is potential inflammation after introduction into the living eye. To investigate their inflammatory responses, the two potential tamponades found to have the most different biocompatibility profiles in our earlier trials (Healaflow® and Bio-Alcamid®) were compared to Balanced Saline Solution (BSS) in the experimental *in vivo* vitrectomy model. The surgical technique was further refined, using only 25-gauge pars plana vitrectomy with subsequent injection of 0.5 - 1 ml of Healaflow®, Bio-Alcamid® (through an 18-gauge incision), or Balanced Saline Solution (BSS). Postoperative clinical evaluation was performed, and the rabbits were sacrificed at 1 day, 1 week, or 1 month. After enucleation, the eyes were examined macroscopically, and prepared for histological examination with routine microscopy and immunohistochemical analysis of CD68 and CD45r0 including the retina and remaining peripheral vitreous. CD45r0 labelling was seen in the Ganglion Cell Layer (GCL) and Inner Nuclear Layer (INL) in both operated and unoperated eyes.

Infiltrating CD68+ cells were seen mainly in eyes with retinal complications such as ruptures or localized retinal detachments. All of the eyes treated with Bio-Alcamid® exhibited increased macroscopic inflammation, and cataract development. One of these eyes sustained endophthalmitis-like inflammation, and the structure of all these retinas were severely affected. Infiltrating CD68+ and large amounts of CD45r0+ cells were found in the vitreous, and in the affected retinas.

Artificial polymer gels have been known to cause foreign body reactions in other tissues, and it is not unlikely that the increased inflammation seen in our Bio-Alcamid®-injected eyes may be of the same origin. The very limited response noted in Healaflow®-injected counterparts, indicates that its composition and structure which is more similar to the natural vitreous might account for its excellent biocompatibility. These findings are congruent with our previous results.

Further experiments were performed with silicone oil, a tamponade commonly used in clinical vitreoretinal surgery (results pending).

As a final conclusion, the study provides an increased understanding of normal and pathological responses which may facilitate the development of more biocompatible vitreous substitutes.


To further explore the possibility of cross-linked hyaluronic acid hydrogels as clinically relevant vitreous substitutes, further studies are planned in the fine-tuned *in vivo* vitrectomy model. A trial with a more cross-linked variant of the previously used Healaflow® in conjunction with a new *in vivo* retinal detachment model is in the planning stages.

**Conclusion**

A need for safer and more biocompatible vitreous substitutes to better treat patients suffering from vitreoretinal disease is clearly at hand. Healaflow®, and related cross-linked hyaluronic acid hydrogels, show promise to fill these needs. Further studies are warranted to further evaluate this group of substances, hopefully leading to new therapies. This project has also produced new methods for studying vitreous substitutes, potentially leading to safer, more economic and refined *in vivo* and *in vitro* animal experiments prior to more elaborate studies.
A new model for in vitro testing of vitreous substitute candidates

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Abstract

Purpose To describe a new model for in vitro assessment of novel vitreous substitute candidates.

Methods The biological impact of three vitreous substitute candidates was explored in a retinal explant culture model; a polyalkylimide hydrogel (Bio-Alcamid®), a two component hydrogel of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PEG) and a cross-linked sodium hyaluronic acid hydrogel (Healaflow®). The gels where applied to explanted adult rat retinas and then kept in culture for 2, 5 and 10 days. Gel-exposed explants were compared with explants incubated under standard tissue culture conditions. Cryosections of the specimens were stained with hematoxylin and eosin, immunohistochemical markers (GFAP, Vimentin, Neurofilament 160, PKC, Rhodopsin) and TUNEL.

Results Explants kept under standard conditions as well as PEG-exposed explants displayed disruption of retinal layers with moderate pyknosis of all neurons. They also displayed moderate labeling of apoptotic cells. Bio-Alcamid®-exposed explants displayed severe thinning and disruption of retinal layers with massive cell death. Healaflow®-treated explants displayed normal retinal lamination with significantly better preservation of retinal neurons compared with control specimens, and almost no signs of apoptosis. Retinas exposed to Healaflow® and retinas kept under standard conditions showed variable labeling of GFAP with generally low expression and some areas of upregulation. PEG-exposed retinas showed increased GFAP labeling and Bio-Alcamid®-exposed retinas showed sparse labeling of GFAP.

Conclusions Research into novel vitreous substitutes has important implications for both medical and surgical vitreoretinal disease. The in vitro model presented here provides a method of biocompatibility testing prior to more costly and cumbersome in vivo experiments. The explant culture system imposes reactions within the retina including disruption of layers, cell death and gliosis, and the progression of these reactions can be used for comparison of vitreous substitute candidates. Bio-Alcamid® had strong adverse effects on the retina which is consistent with results of prior in vivo trials. PEG gel elicits reactions similar to the control retinas whereas Healaflow® shows protection from culture-induced trauma indicating favorable biocompatibility.

Keywords Vitreous substitute · Immunohistochemistry · Retinal culture · Vitreoretinal surgery · Hyaluronic acid · Polyethylene oxide · Polyal kylimide

Introduction

Vitrectomy is a common procedure for several eye disorders capable of severely impacting the vision of affected patients and has an important role in the treatment of conditions such as rhegmatogenous retinal detachment, severe diabetic retinopathy, penetrating ocular trauma, macular holes and epiretinal membranes. The removal of vitreous tissue during vitreoretinal surgery mandates its replacement, either in the form of water or various tamponading agents. The compounds currently in widespread clinical use such as balanced salt
solutions, gases, silicon oils and perfluorocarbon liquids all have considerable disadvantages, with complications such as cataract formation, uveitis, increased intraocular pressure [1] and cytotoxicity [2, 3]. Further, current tamponading agents are either resorbed spontaneously after a few weeks or are not suitable for long-term use [4–9], and may require strict body positioning postoperatively.

The search for improved vitreous substitutes has been ongoing since the early days of the 20th century [10]. Early attempts were made to transplant animal and human vitreous [11] and investigations have been made into numerous semi-synthetic [12–14] and synthetic [15] molecules, although few of them have reached a clinical setting and none have fulfilled the requirements for long-term biocompatibility.

Traditionally the interactions of vitreous substitutes with eye tissues have been studied in various animal models in vivo. Such trials are, however, costly, time consuming and might be considered ethically problematic. In some cases in vivo experiments have been precluded by preclinical toxicological assays, mainly targeting apoptosis in cultures of cells from tissues outside the eye [16, 17], isolated retinal pigment epithelium (RPE) cells [18, 19] or dissociated cells from embryonal retinas [20]. The validity of these findings in relation to a clinical setting is, however, unclear since they represent a large transitional step regarding the impact on the adult neuroretinal sheet [21]. Therefore, a means to investigate the biological impact of vitreous substitutes more similar to the in vivo situation is desirable.

For this paper we wanted to explore a novel in vitro model for investigating the biological impact of vitreous substitutes on the neuroretina. To this end we have used the well-established retinal explant model to study three polymer hydrogels of different chemical composition that theoretically may be considered as potential vitreous substitutes; 1) Cross-linked hyaluronic acid (Healaflow®), clinically used in glaucoma surgery [22, 23]; 2) Poly (ethylene glycol) (PEG), widely used in different biochemical applications [24, 25]; and, 3) Polyalkylimide (Bio-Alcamid®), clinically used in reconstructive surgery [26–29].

Materials and methods

Three vitreous substitute candidates were investigated in the retinal explant culture model: a cross-linked sodium hyaluronic acid (22.5 mg/ml) hydrogel (Healaflow®); a two component hydrogel of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PEG) and a polyalkylimide hydrogel (Bio-Alcamid®). The gels where applied to explanted adult rat retinas and then kept in culture for 2, 5, and 10 days in vitro (DIV). Gel-exposed explants were compared with explants incubated under standard conditions (medium only). Cryosections of the specimens were stained with hematoxylin and eosin, immunohistochemical markers (GFAP, Vimentin, PKC, NF160, Rhodopsin) and TUNEL.

Animals

Retinas from adult Sprague–Dawley rats were used. All procedures and animal treatment were in accordance with the guidelines and requirements of the government committee on animal experimentation at Lund University and with the Association for Research in Vision and Ophthalmology (ARVO) statement on the use of animals in ophthalmic and vision research.

Gels

Healaflow® (Anteis S.A., Plan Les Ouates, Switzerland) is a commercially available translucent hydrogel, clinically used in glaucoma filtering surgery as a space-filler and to limit postoperative fibrosis [22, 23]. The hydrogel consists of over 97% water, sodium hyaluronic acid (22.5 mg/ml) of non-animal origin cross-linked with BDDE (1,4-Butanediol diglycidyl ether), and phosphate- and NaCl-salts to maintain a physiological pH (7.0) and osmolarity (305 mOsm/kg). The estimated specific gravity is circa 1.03, and the refractive index i=1.341.

A custom made two component cross-linked hydrogel (PEG) consisting of 20 wt.% poly (ethylene glycol) in phosphate buffered saline (PBS) was prepared by mixing PEGDA in PBS into ETTMP-1300 in PBS [30]. PEG is a synthetic water-soluble polymer approved by the FDA for biomedical use in different applications including injectable hydrogels. It has been investigated for use in intravitreal drug delivery, repair of scleral incisions and the sealing of retinal breaks in retinal detachment surgery [24, 25].

Bio-Alcamid® (Polymekon, Brindisi, Italy) is a commercially available clear hydrogel in clinical use as tissue filler for plastic and reconstructive surgery, mainly for lipoatrophic and posttraumatic conditions. The hydrogel consists of approximately 4% reticulated polyalkylimide and approximately 96% non-pyrogenic water (pH 6.9), it contains no free monomers and is considered physically and chemically stable [29]. In vivo a collagen capsule surrounding the implanted Bio-Alcamid® is formed.

Tissue handling and culture procedure (Fig. 1)

The rats were euthanized with CO2 with subsequent decapitation, enucleation and immediate immersion of the eyes in an ice-cold CO2-independent medium (Gibco, Paisley, UK). The neuroretinas were dissected from the retinal pigment epithelium (RPE) and the vitreous with fine forceps, and either half or the entire neuroretinas were subsequently explanted on
to culture plate inserts (Millicell Isopore-PCF 0.4 μm, 30 mm; Millipore, Billerica, ME) with the photoreceptor layer against the membrane, and covered by 50–100 μl gel (Healaflow®, PEG, or Bio-Alcamid®) depending on the size of the explant. The explants were cultured in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium–l-glutamine (Gibco) supplemented with 10% fetal calf serum, with a drop of enriched medium applied directly onto the gels to ensure saturation. The cultures were also supplemented with 2 mM l-glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin (Sigma-Aldrich, St Louis, MO), and the retinas were kept at 37 °C at 95% humidity and 5% CO2. Four explants in each group (standard conditions, Healaflow®, PEG, and Bio-Alcamid®) were kept in culture for 2 days and six explants in each group were kept for 5 or 10 days, with exchange of half the culture medium after 3, 5, 7 and 9 days. No exchange of gel was made during the change of medium.

Immunohistochemistry

In preparation for further histological studies the explants were fixed for 1 h in 4% formalin (pH 7.3) in 0.1 M Sørensen phosphate buffer (PB). The specimens were then washed with 0.1 M Sørensen PB; this was repeated with the same solution containing sucrose of increasing concentrations (5–25 %). Specimens were sectioned to 12 μm on a cryostat and every tenth slide was stained with hematoxylin and eosin according to standard procedures.

For immunohistochemical staining sections were washed at room temperature with 0.1 M of sodium PBS (pH 7.2) with 0.1% Triton X–100 (PBS/Triton), and thereafter incubated overnight at 4 °C with antibodies against the following antigens; Rhodopsin [rod photoreceptors] (Rho4D2, a kind gift from Prof. R.S. Molday, Vancouver, Canada; monoclonal, diluted 1:100), phospho-protein kinase C [PKC, rod bipolar cells] (K01107M; Cell Signaling, USA; diluted 1:200), Neurofilament 160 KDa [NF1 60, ganglion and horizontal cells] (clone NN18; Sigma, St. Louis, MO, USA; diluted 1:500), glial fibrillary acidic protein [GFAP, activated Müller cells] (clone G-A-5; Millipore, Sundbyberg, Sweden; diluted 1:200 with PBS/Triton with 1% bovine serum albumin) and vimentin [Müller cells] (Chemicon, USA; 1: 500). After incubation with the antibodies and rinse with PBS/Triton the slides were incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 45 min, rinsed and mounted in an anti-fading mounting media (Vectashield, Vector laboratories, Inc., Burlingame, CA, USA). Negative controls were obtained by performing the same procedure as above but without any primary antibodies. Antibodies are summarized in Table I. For identification of apoptotic cells a commercial terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay system with fluorescein-conjugated dUTP (Boehringer Mannheim, Mannheim, Germany) was used on the retinal sections according to the manufacturers instruction.

Results

Retinal explant cultures

All gels (Healaflow®, PEG and Bio-Alcamid®) could successfully be applied to the explanted retinal tissue. Healaflow® and PEG formed even films over the retinal explants whereas Bio-Alcamid® retained a thick, uneven
texture that did not cover the explants completely even after a prolonged time. The PEG gel was found to benefit from a 20 min incubation time prior to administration of the medium, allowing for some gelation and preventing dissolution. The gels remained translucent and could be visualized at every exchange of the medium and were confirmed to be macroscopically saturated with the colored medium by means of visual inspection. Two of the explant-cultures suffered infection and were excluded from further analysis.

Cytoarchitecture and cell death (Fig. 2)

After two DIV hematoxylin- and eosin-stained sections of explants kept under control conditions as well as PEG-exposed explants displayed an abnormal retinal lamination with a wavy appearance of the outer nuclear layer (ONL). The ONL also displayed variable thickness, displacement towards the inner retina and moderate pyknosis. Inner retinal layers displayed some variability in total thickness and moderate pyknosis. Healaflow®-treated explants showed almost normal retinal lamination with significantly better preservation of retinal neurons compared with control specimens, whereas Bio-Alcamid®-exposed explants displayed a highly variable cytoarchitecture with severe thinning and disruption of all retinal layers in most parts, and a less disrupted structure in minor areas. TUNEL labeling at 2 DIV demonstrated no or almost no apoptotic cells with explants kept under control conditions, with Healaflow® and with PEG, and some apoptosis with explants cultured with Bio-Alcamid®. After 5 and 10 DIV a progressive increase in pyknosis and laminar disruption was seen in all groups. Retinas kept under standard conditions, and especially with Healaflow®, exhibited less pyknosis and laminar disruption than those treated with PEG and Bio-Alcamid®. TUNEL labeling of 5 DIV explants kept under control conditions and those subjected to PEG displayed moderate signs of apoptosis. Healaflow®-treated retinas showed almost no TUNEL labeling whereas explants treated with Bio-Alcamid® displayed massive cell death. At 10 DIV intense TUNEL labeling was observed in explants cultured under standard conditions, low labeling with Healaflow®-treatment and very low labeling in the PEG-and Bio-Alcamid®-treated cultures.

Rod photoreceptors (Fig. 3)

Rhodopsin-labeled photoreceptor cells in standard cultures displayed high intensity labeling of the outer segments (OS) and in the outer plexiform layer (OPL), with mild intensity labeling present in the ONL. Similar patterns of labeling were seen at 2 and 5 DIV. At 10 DIV stronger labeling was seen in the ONL. The Rhodopsin labeling pattern of Healaflow®- and PEG-exposed explants was comparable to the standard conditions. Table I Specification of immunochemical markers

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<th>Species</th>
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<td>GFAP</td>
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<td>Astrocytes, activated Müller cells</td>
<td>Mouse monoclonal</td>
<td>1200</td>
<td>Chemicon International, CA, USA</td>
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<tr>
<td>Neurofilament 160 KDa (NF160)</td>
<td>Anti-Neurofilament 160 clone NN18</td>
<td>Ganglion and horizontal cells</td>
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<td>1500</td>
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<tr>
<td>PKC</td>
<td>Phospho-PKC (pan)</td>
<td>Rod bipolar cells</td>
<td>Rabbit polyclonal</td>
<td>1200</td>
<td>Cell Signaling, Beverly, MA, USA</td>
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<td>Rho4D2</td>
<td>Rod photoreceptor</td>
<td>Rod photoreceptor</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
<td>Kind gift of Prof. RS Molday, Vancouver, Canada</td>
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<td>Anti-mouse</td>
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<tr>
<td>FITC</td>
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<td>Anti-rabbit</td>
<td>Goat</td>
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<td>Southern Biotechnology Associates, AL, USA</td>
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Fig. 2 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with standard conditions (CTRL), Healaflow® (HF), PEG-gel (PEG) and Bio-Alcamid® (BA). Hematoxylin and eosin staining (top rows), and TUNEL staining (bottom rows). Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=25 μm
control. Bio-Alcamid® explants displayed intense labeling of the entire ONL already at 2 DIV.

Inner retinal cells (Fig. 4)

PKC labeling for rod bipolar cells at 2 DIV displayed a high variability with the most intense labeling towards the peripheral edge of the control explants. In 5 DIV specimens only a few PKC-labeled cell bodies were found whereas 10 DIV specimens did not show any remaining rod bipolar cells. In Healaflow®-treated specimens at 2 DIV a few PKC-labeled rod bipolar cells were found, but in older explants no such cells were found. PEG and Bio-Alcamid® explants did not display any PKC-labeled rod bipolar cells.

Neurofilament 160-labeled ganglion cells were seen in all retinal cultures with no clear differences between the different tested gels. No difference was observed between different incubation times.

Müller cells (Fig. 5)

GFAP labeling, indicative of Müller cell activation, showed very low intensity in most parts of the control retinas at 2 DIV but intense labeling was present in astrocytes located in the innermost retina. A generally low labeling intensity was seen at 5 DIV with some areas of moderate to high labeling of Müller cells (shown in Fig. 4). At 10 DIV some areas of moderate labeling was seen with mostly fragmentary labeling having a tortuous appearance of the Müller cell fibers. Healaflow®-subjected retinas displayed patterns similar to those of the control retinas at all timepoints, although there
was a tendency towards a slightly lower labeling intensity at 5 DIV.

The retinas exposed to PEG and Bio-Alcamid® displayed a high labeling intensity in the inner retina with labeled Müller cell fibers occasionally reaching the ONL at 2 DIV. After 5 DIV moderate, variable expression both in the inner retina and in fibrils was exhibited on the PEG exposure. Bio-Alcamid®-exposed retinas exhibited low labeling intensity, almost exclusively in the inner retina. At 10 DIV cultures with PEG showed moderate, variable expression and those cultured with Bio-Alcamid® displayed only weak labeling present in the inner retina.

Vimentin labeling of Müller cell cytoskeletons was present in fibers through the inner parts of the retina, in some areas through to the ONL, with some labeling in the innermost retina. No significant differences were seen between the different groups but increased hypertrophy and disorganization of Müller cell fibers was seen over time with the labeling pattern appearing almost granular at later time points.

Discussion

Summary

In this study a new in vitro model for evaluating the effect of potential vitreous substitutes on adult neuroretinal sheets was explored. Three potential candidates were evaluated and compared to retinal explants cultured under standard conditions. Clear differences were seen between the groups with similar effects observed in explants cultured under standard conditions and with Healaflow®, and more degenerative findings in cultures with PEG and, particularly, Bio-Alcamid®. The relative degenerative morphological and immunohistochemical changes for the different gels compared to standard conditions are summarized as qualitative compound scores in Table 2.

The in vitro model

Research into novel vitreous substitutes has important implications for both medical and surgical vitreoretinal disease. An in vitro assay analyzed using immunohistochemistry and morphological stainings can determine the biocompatibility and safety of potential vitreous substitutes. This may provide better predictions of the effects of novel substances on the retina than the traditional, more simplistic in vitro assays currently in use [16–19, 21, 31].

The in vitro model presented here provides a method of biocompatibility testing prior to more costly and cumbersome in vivo experiments [20]. In retinal explant cultures under standard conditions there are several well-characterized reactions easily observable as early as 3 or 4 DIV [32–34]. These reactions include gliosis and neuroretinal degeneration and can be visualized by GFAP upregulation, disruption of the cell layers and the labeling of apoptotic cells. Using these reactions elicited by the explant culture system under standard conditions and comparing them to different vitreous substitute candidates indicates the biocompatibility of the substances in vivo.

Our previous results and our hypothesis

The vitreous is often simplistically seen as a mere space filler inside the eye bulb. There is, however, evidence of a more intricate and purposeful composition [10] with important physiological implications on the micro-milieu of the retina including the upkeep of salt and nutrient gradients, physical support and more [35, 36]. An ideal vitreous substitute would replicate these influences on the neuroretina and surrounding tissues as well as provide a tamponading effect after vitrectomy [10].

In two recent papers our group investigated two promising potential intravitreal substitutes in an in vivo rabbit model: Polyalkylimide (Bio-Alcamid®) [37] and a poly (ethylene glycol) (PEG) hydrogel [38].

Bio-Alcamid® is a translucent hydrogel with high biocompatibility [26, 27] used in plastic surgery and in clinical use forms a surrounding collagen capsule giving it a degree of isolation from the surrounding tissue [28]. The synthetic polymer hydrogel PEG is used in different biomedical application, has been tested for intravitreal administration of drugs [24, 39] and is FDA approved for use intravitreally. The in vivo trials showed favorable biocompatibility but inadequate stability in vivo using PEG where the substance was largely tolerated in vivo. However, Bio-Alcamid® displayed suitable physical properties but caused severe functional and morphological retinal damage with increased GFAP expression and cell death (TUNEL) [37].

The use of derivates of sodium hyaluronic acid in vitreoretinal surgery predates their ubiquitous use in cataract and anterior segment surgery [12, 14, 40–42], but their use in a clinical setting has been limited mainly due to concern about short term side effects and retention time [41, 43]. Healaflow® is a commercially available compound consisting of a cross-linked sodium hyaluronic acid...
hydrogel and is FDA approved for use in glaucoma surgery [22, 23]. The composition of Healaflow® is akin to natural vitreous: a reinforced hydrogel of hyaluronic acid with similar physical properties and thus considered a plausible candidate for vitreous substitution.

In vivo vs. in vitro: our earlier results and others

It seems to us that a good correlation exists between the results of this in vitro explant culture system and earlier results for all the tested substances.

In this setting retinal explants cultured with Healaflow® compare very well to specimens cultured under standard conditions and even seem to lessen the trauma caused by the culture process. This is consistent with the excellent biocompatibility of hyaluronic acid seen in other studies [18, 19]. Hyaluronic acid is one of the main constituents of natural vitreous and is consistently well-tolerated in different biomedical applications. Healaflow® may exert a protective effect from culture-induced trauma on the retinal explants by providing a more physiologically similar microenvironment in vitro. Additionally, the positive effect on the retina could be due to biomechanical factors via physical interaction from the gel that might prevent retinal folds and keep the explants under tension. This is a factor that previously has been showed to favorably affect retinas in vitro [44].

PEG gel elicits reactions similar to the control retinas with comparable changes in the cytoarchitecture but with earlier, more intense TUNEL labeling, consistent with previous in vivo findings [38]. In the retinal explant cultures with the longest duration (10 DIV) there was a decrease in the amount of apoptotic cells observed at earlier time points. This may be due to a loss of viable cells as cell death occurred earlier than for Healaflow® and standard conditions, indicating a stronger adverse reaction to these gels than what is caused by the culture procedure.

Bio-Alcamid® caused severe retinal damage in vivo [37] and negatively affected the morphology of cells and cell layers, induced cell death and induced GFAP upregulation very early in vitro. Some of the variability in cytoarchitecture for retinas treated with Bio-Alcamid® might have been due to uneven coverage of the gel. The adverse effects may in part be influenced by uneven exposure to the medium, but cytotoxic effects from the gel itself are likely to play a part in this process. The explanted retinas were less affected in minor areas that may not have been in direct contact with the gel, although this is difficult to discern due to the loss of gel in the preparation and sectioning procedures. This is in accordance with previous studies that demonstrated pathological changes in the retina in vivo, primarily in parts more likely to have been in direct contact with the gel [37], suggesting at least in part a toxic or immunological response. Recently, clinical use of Bio-Alcamid® in reconstructive surgery has become increasingly controversial due to late complications such as inflammation, infection and excessive capsule formation [45–48].

**Conclusion**

The retinal explant assay described in this paper has the potential to be a useful tool for preliminary study of vitreous substitute candidates prior to more costly and time-consuming in vivo testing. In addition, it may reduce the need for laboratory animals and limit the severity of the experiments from an ethical standpoint by excluding unfit substances from further testing, thereby providing refinement of the tests. In vivo tests will still be essential before testing on human subjects but this assay may minimize the translational step which would prove valuable and beneficent in vetting out unsuitable biomaterials.

A need for better vitreous substitutes still remains and more suitable substances would be highly valuable. Healaflow® and, to a lesser extent, PEG seem to be promising candidates for further development and further in vivo testing of these and similar substances is clearly indicated.

**Acknowledgment**

This work was supported by: The Faculty of Medicine, University of Lund, The Swedish Research Council, The Princess Margareta’s Foundation for Blind Children, The Wallenberg Foundation. T.M.O. was supported by a Sir General John Monash Scholarship. Some of this work was sponsored by a gift to MIT by the In Vivo Therapeutics Corporation.

Thanks to Karin Arnér for excellent technical support and Linnéa Taylor for valuable input on the manuscript.

**Financial disclosures** None.

**Statements** The authors have full control of all primary data, available for review by Graef’s Archive for Clinical and Experimental Ophthalmology upon request. The “Principles of laboratory animal care” (NIH publication No. 85–23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised

---

**Table 2** Relative compound score for the degenerative retinal changes for the different gels compared to standard conditions ranging from – to ++

<table>
<thead>
<tr>
<th>Gels</th>
<th>Cytoarchitecture and cell death</th>
<th>Rod photoreceptors</th>
<th>Inner retinal cells</th>
<th>Müller cell activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healaflow®</td>
<td>_</td>
<td>0</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>PEG</td>
<td>+</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Bio-Alcamid®</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
References

glycol) sols as vitreous substitutes in an experimental vitrectomy model in rabbits. Acta Biomater 7:936–943


A cross-linked hyaluronic acid hydrogel (Healaflow®) as a novel vitreous substitute

Henrik Barth1 · Sven Crafoord2 · Sten Andréasson1 · Fredrik Ghosh1

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Abstract

Purpose Vitrectomy requires the substitution of the natural vitreous, as well as tamponading of retinal breaks. Clinically available alternatives such as gas and silicone oil have side effects such as inflammation, secondary glaucoma, cataract, and a need for head posturing. In this study, a hydrogel of cross-linked sodium hyaluronic acid (Healaflow®) is evaluated for use as a novel vitreous substitute.

Methods A combined 25-20-gauge pars plana vitrectomy with posterior vitreous detachment was performed in the right eye of twelve pigmented rabbits, with subsequent injection of approximately 1 ml Healaflow®. Clinical evaluation, measurement of intraocular pressure (IOP), and full-field ERG were performed postoperatively. The rabbits were sacrificed at different time-points between 42 and 105 days. After enucleation, the eyes were examined macroscopically, photographed, and prepared for histological examination with routine microscopy and immunohistochemistry.

Results Healaflow® was successfully used with standard surgical procedures and remained translucent but did lose most of its viscosity during the postoperative period. One rabbit was lost due to unrelated causes. In two eye atrophic partial retinal detachments were seen, and in two eyes significant cataract developed due to intra-operative complications. ERG-recordings revealed no toxic effect on rod or cone function. Routine microscopy and immunohistochemistry demonstrated normal morphology with some Müller cell activation (up-regulation of glial acidic fibrillary protein, GFAP) compared to unoperated eyes and no significant DNA-fragmentation (TUNEL-assay).

Conclusions Healaflow® did not affect retinal morphology or function negatively during long-term use as a vitreous substitute, making it highly interesting in this setting. An estimated retention time of a few weeks suggests potential for use as a short-term tamponade. Future work will include an increased ratio of cross-linking to prolong the structural integrity of the gel.

Keywords Vitreous substitute · Vitreoretinal surgery · Hyaluronic acid · Immunohistochemistry

Introduction

Vision threatening disorders such as rhegmatogenous retinal detachment, severe diabetic retinopathy, penetrating ocular trauma, macular holes, and epiretinal membranes often require surgical intervention such as vitrectomy. An infusion of Balanced Saline Solution (BSS) is used during the surgery, but it is often necessary to replace the BSS with a tamponading agent to prevent fluid from re-entering retinal breaks during the healing process. The clinically used substances, such as gases and silicone oils, have several unwanted side effects. Among them are complications such as low vision, cataract formation, uveitis [1], increased intraocular pressure [2], and cytotoxicity [3–5]. These substances are either resorbed by the body after a relatively short time, or are otherwise unsuited for long-term use and therefore removed [6–9], and may require strict posturing of the head for an extended period of time postoperatively. Additionally, there is evidence that physiological changes in the vitreous could
play a role in different pathological processes such as post-
vitrectomy cataract formation [10], and glaucoma [11]. These
changes include altered diffusion gradients of nutrients and
other biologically important substances [12], and could feasi-
ibly be alleviated by improved vitreous substitutes.

Clearly, there is a need for more suitable vitreous substitutes,
both to enhance the biocompatibility and lessen the side effects,
and possibly to allow for safe long-term substitution. This is
especially true in complex cases that require silicone oils, which
are considerably more prone to induce side effects than the more
extensively used gases, although a vitreous substitute that would
permit faster visual rehabilitation and less patient discomfort
than the gases would also be highly beneficial. The task to
devising an optimal vitreous substitute is demanding, requiring
consideration of a variety of parameters such as clinical usability,
physical properties, biomechanics, and the biochemical micro-
milieu. Studies in vitreous substitution have been ongoing since
the early 20th century, with experimental use of substances rang-
ing from transplanted animal and human vitreous to more recent
semi-synthetic [13–15], and synthetic [16] substances. So far,
one of these substances have proved more useful than the
clinically available alternatives.

A potentially fruitful way to devise a substance able to
fulfill these requirements is to mimic the healthy vitreous.
The natural vitreous is a hydrogel consisting of hyaluronic
acid reticulated with collagen fibers, thus reinforcing the gel.
A similarly structured synthetic gel, such as a cross-linked
hyaluronic acid hydrogel, could provide beneficial physiological
properties [17]. Healaflow® is a commercially available
substance of this type, developed for glaucoma surgery. In a
previous in vitro study it was tolerated well by retinal explant
cultures [18], and it seems to harbor potential for use as a
novel vitreous substitute. In this in vivo study, we explore
Healaflow® as a novel vitreous substitute in a previously
established rabbit vitrectomy model [19, 20].

Materials and methods

Animals

Twelve pigmented rabbits, aged 4 months, were used in the
experiments. All proceedings and animal treatment were in
accordance with the guidelines and requirements of the gov-
ernment committee on animal experimentation at Lund
University and with the ARVO (The Association for
Research in Vision and Ophthalmology) statement on the
use of animals in ophthalmic and vision research.

Gel properties

Healaflow® (Anteis S.A., Plan Les Ouates, Switzerland) is a
commercially available transparent hydrogel, clinically used
in glaucoma filtering surgery as a space-filler, and to limit
postoperative fibrosis [21, 22]. The hydrogel consists of over
97 % water, sodium hyaluronic acid (22.5 mg/ml) of non-
animal origin cross-linked with BDDE (1.4-Butanediol
diglycidyl ether), and phosphate- and NaCl-salts to maintain
physiological pH (7.0) and osmolarity (305 mOsm/kg). Estimated
specific gravity is circa 1.03, and refractive index
i = 1.341.

Surgery

Experienced surgeons performed all surgical procedures un-
der general anesthesia, where a combination of ketamine
(35 mg/kg) and xylazine (5 mg/kg) was given intramuscularly.
The right eyes of the rabbits were vitrectomized, while the left
eyes served as controls. The pupils of the right eyes were
dilated with cyclopentolate (1 %) and phenylephrine (10 %),
and anesthetized with topical tetracaine (0.5 %) just before
surgery. Sclerotomies were made 1 mm posterior to limbus
with two transconjunctival 25 G trocars for infusion and
illumination purposes, and either a transconjunctival 25 G tro-
car or a conjunctival incision and subsequent 20 G sclerotomy
for the main instrument. Balanced salt solution (BSS,
Endosol™, Abbott Medical Optics) was used as a continuous
infusion. A BIOM 90-D lens (Oculus) and a standard endo-
iluminating probe were used for visualization. With the
Accurus Surgical System™ (Alcon, Fort Worth, TX, USA)
and a vitreous cutter (Innovit®, Alcon) posterior vitreous
detachment (PVD) was initiated by positioning the vitrectomy
probe at the margin of the disk, and applying suction and
traction with the vitrectomy probe. PVD was, if possible, vi-
sually confirmed, and a central vitrectomy performed, leaving
peripheral parts of the vitreous intact due to the increased risk
of traumatic cataract with the comparatively large lens of the
rabbit. After fluid-air exchange, approximately 1 ml
Healaflow® was injected through a 25 G needle. The
sclerotomies and conjunctiva were sutured if needed, and
25 mg gentamicin and 2 mg betamethasone were administered
subconjunctivally. No other postoperative treatment was
given.

Postoperative handling

Clinical evaluation with ophthalmoscopy, Retcam™ photog-
raphy, intraocular pressure (IOP) measurement (Tono-
Pen®), and full-field ERG was performed postoperatively
at intervals up to 105 days. The rabbits were sacrificed at
different time-points between 42 and 105 days. After enu-
cleration, the eyes were examined macroscopically,
photographed, and prepared for histological examination
and immunohistochemistry.
**Electrophysiology**

Full-field electroretinograms (ERG) from the right eyes were obtained preoperatively, at 1 and 3 months after surgery for six of the rabbits, and at 1 month postoperatively for the rest of the rabbits. A Nicolet Viking® analysis system (Nicolet Biomedical Instruments, Madison, Wisconsin) was used for the recordings as described previously [23, 24]. The rabbits were sedated with an intramuscular injection of Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml). Pupil dilation was performed with cyclopentolate (1 %), and topical anesthesia applied. After 30 minutes of dark adaptation, a Burian-Allen bipolar ERG contact lens electrode was applied on the cornea with 2 % methylcellulose lubrication, and a subcutaneous ground electrode attached to the neck. Responses were obtained with a wide band filter (3 dB at 1 Hz and 500 Hz). Stimulations were performed with single full-field flashes (30 μs), with dim blue light (Wratten filters #47, 47A and 47B), and white light (0.8 cd.s/m2) without a background. Cone responses were obtained using 30 Hz flickering white light (0.8 cd.s/m2) averaged from 20 sweeps without a background light. The luminances of the different light stimuli refer to the light reflected from the Ganzfeld sphere.

**Tissue handling and immunohistochemistry (Table 1)**

The rabbits were euthanized at different time-points between 42 and 105 days and the eyes were enucleated, dissected, and fixation performed for 1 h in 4 % formalin, pH 7.3 in a 0.1 M Sørensen’s phosphate buffer (PB). The specimens were washed with 0.1 M Sørensen’s PB, and then washed again using the same solution containing increasing concentrations of sucrose (5–25 %). The specimens were cryosectioned with a section thickness of 12 μm, and every 10th slide was stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry, sections were washed three times in room temperature with 0.1 M of sodium phosphate-buffered saline pH 7.2 (PBS) with 0.1 % Triton X-100 (PBS/Triton), and incubated with PBS/Triton with 1 % bovine serum albumin (BSA). The sections were thereafter incubated overnight at +4 °C with antibodies (diluted in PBS/Triton with 1 % BSA) against the following antigens: Rhodopsin [rod photoreceptors] (Rho4D2, a kind gift from Prof. R.S. Molday, Vancouver, Canada; monoclonal, diluted 1:100), phospho-protein kinase C [PKC, rod bipolar cells] (K01107M; Cell Signaling, USA, diluted 1:200), and glial fibrillary acidic protein [GFAP, activated Müller cells] (clone G-A-5; Millipore, Sundbyberg, Sweden, diluted 1:200). The slides were rinsed with PBS/Triton, and incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies (Sigma-Aldrich, St.Louis, MO, USA, diluted 1:200) for 45 min, rinsed again, and mounted in anti-fading mounting media (Vectashield, Vector laboratories, Inc., Burlingame, CA, USA). Negative controls were obtained by performing the same procedure without primary antibodies. The antibodies are summarized in Table 1. For identification of apoptotic cells, a commercial terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay system with fluorescein-conjugated dUTP (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions.

**Results**

**Macroscopic findings (Fig. 1 and Tables 2 and 3)**

Healaflo® was easily incorporated in the standard surgical procedure and could successfully be injected through small gauge needles. During the surgeries, iatrogenic retinal breaks occurred in four cases and the lens was accidentally touched in two cases. One rabbit was lost due to unrelated causes. No signs of excessive conjunctival swelling or uveitis were seen. Postoperative IOP (15–25 mmHg, see Table 3) was slightly elevated compared to earlier results [19]. In the two

---

**Table 1** Specification of immunohistochemical markers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody name</th>
<th>Target structure</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>Anti-glial fibrillary acidic protein</td>
<td>Astrocytes, activated Müller cells</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
<td>Chemicon International, CA, USA</td>
</tr>
<tr>
<td>PKC</td>
<td>Phospho-PKC (pan)</td>
<td>Rod bipolar cells</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Cell Signaling, Beverly, MA, USA</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>Rho4D2</td>
<td>Rod photoreceptor</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
<td>Kind gift of Prof. RS Molday, Vancouver, Canada</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Antibody name</td>
<td>Target</td>
<td>Species</td>
<td>Dilution</td>
<td>Source</td>
</tr>
<tr>
<td>FITC</td>
<td>Anti-mouse IgG FITC conjugate</td>
<td>Anti-mouse</td>
<td>Goat</td>
<td>1:200</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>FITC</td>
<td>Goat Anti-Rabbit IgM + IgG (H + L chain specific)</td>
<td>Anti-rabbit</td>
<td>Goat</td>
<td>1:200</td>
<td>Southern Biotechnology Associates, AL, USA</td>
</tr>
</tbody>
</table>
eyes with traumatic cataracts, lens material was observed in the vitreous cavity, and iatrogenic partial retinal detachments were seen. These retinal detachments stayed partial for the duration of the follow-up. None of the other cases of operative complications developed retinal detachment. The untreated lesions were seen to heal with pigmented scar formation. No intact gels were visible after dissection, although some gel remnants more viscous than water were seen.

Table 2  Summary of operative and postoperative data

<table>
<thead>
<tr>
<th>Case</th>
<th>Follow-up time (days)</th>
<th>Preoperative complications</th>
<th>Cataract</th>
<th>Postop status (macro- and microscopically)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>Two retinal lesions</td>
<td>No</td>
<td>Pigmented lesions. Normal histology, no upregulation of GFAP, PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>2</td>
<td>3 (deceased)</td>
<td>None</td>
<td>No</td>
<td>N/a</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>One retinal lesion</td>
<td>Slight</td>
<td>Pigmented lesions. Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>4</td>
<td>105</td>
<td>None</td>
<td>No</td>
<td>Normal histology, no upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>5</td>
<td>105</td>
<td>None</td>
<td>No</td>
<td>Macroscopically normal. Small RD in histology, upregulation of GFAP in some areas. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>6</td>
<td>105</td>
<td>One retinal lesion</td>
<td>Slight</td>
<td>Macroscopically normal. Small RD in histology, upregulation of GFAP in some areas. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>None</td>
<td>No</td>
<td>Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>Traumatic cataract</td>
<td>Severe</td>
<td>Cataract, lens material in vitreous cavity. Limited retinal detachment (RD) with retinal degeneration. Moderate upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>One retinal lesion</td>
<td>No</td>
<td>Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>None</td>
<td>No</td>
<td>Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>11</td>
<td>56</td>
<td>None</td>
<td>No</td>
<td>Normal histology, minimal upregulation of GFAP. PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
<tr>
<td>12</td>
<td>43</td>
<td>Traumatic cataract</td>
<td>Severe</td>
<td>Cataract, lens material in vitreous cavity. Limited retinal detachment (RD) with retinal degeneration. Moderate upregulation of GFAP PKC and Rhodopsin normal. TUNEL negative.</td>
</tr>
</tbody>
</table>
Electrophysiology (Fig. 2)

Full-field ERG-recordings from the vitrectomized rabbits were reproducible and easily detected. There were no observed changes in the amplitudes either of the isolated rod (Dim blue light stimulation), mixed rod and cone (White light), or isolated cone (Flickering light) responses 1 and 3 months after surgery compared to preoperative ERGs.

Histology and immunohistochemistry (Fig. 3 and Table 2)

Routine microscopy with hematoxylin and eosin staining demonstrated normal morphology. The previously seen iatrogenic retinal detachment and retinal lesions were also observable during light microscopy. In two cases, signs of limited retinal detachments were seen although the retina had been deemed attached during the gross examination of the enucleated eyes.

GFAP (glial acidic fibrillary protein) labeling showed minimal signs of Müller cell up-regulation compared to unoperated eyes. In the eyes with traumatic cataracts and major retinal detachments, the GFAP expression was more prominent. No apoptotic cell labeling was observed in any of the eyes using the TUNEL-assay. Labeling with PKC (rod bipolar cells) and Rhodopsin (photoreceptor cells) revealed no differences compared to unoperated eyes.

Discussion

The search for an ideal vitreous substitute has been constantly ongoing since the early days of ophthalmology. Many different approaches have been tried with varying success, but due to the demanding task of combining a clinically usable substance with the correct physical and biochemical properties, there has been no definitive breakthrough so far. The clinically available substances all have considerable drawbacks, including side effects such as retinal toxicity, cataract, secondary glaucoma, and uveitis.

One of the reasons for the difficulties in devising a well-tolerated vitreous substitute is the complex and highly organized environment inside the eye globe. The vitreous cavity has traditionally been viewed in a highly simplified manner, as a space merely filled with a homogenous and inert gel, the vitreous. This simplistic view has gradually changed, and evidence now suggests that the vitreous is highly organized and more physiologically active than previously thought [25, 26]. One of the main functions of the vitreous is to uphold gradients of important nutrients such as oxygen [27], and other substances such as VEGF [10]. After vitrectomy, this environment is inevitably changed, and the addition of a vitreous substitute will further change the properties of the physiological milieu [10]. To prevent fluid from entering retinal breaks a tamponading effect is desirable, mandating close contact with the retina and other sensitive structures. Naturally, this places high demands on the biocompatibility of the substances used as vitreous substitutes.

In two previous papers, we have presented our experience with two potential vitreous substitutes in an in vivo rabbit model, Polyalkylimide (Bio-Alcamid®) and a poly(ethylene glycol) (PEG) hydrogel. In our study Bio-Alcamid® proved to have many suitable properties but caused severe retinal damage [19]. The PEG hydrogel, on the other hand, displayed an acceptable biocompatibility but had an impractically short retention time [20].

Hyaluronic acid is one of the main constituents of the natural vitreous, and related substances have a long history as candidates for vitreous substitutes, predating their routine usage in anterior segment surgery [13, 15, 28]. Due to limited retention time and short-term side effects [29, 30], none of

---

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Postoperative intraocular pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>35 days in vivo (DIV)</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>N/a</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
</tr>
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<td>6</td>
<td>18</td>
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<td>7</td>
<td>25</td>
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<td>8</td>
<td>15</td>
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<td>9</td>
<td>16</td>
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<td>10</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>12</td>
<td>N/a</td>
</tr>
</tbody>
</table>

Fig. 2 Electrophysiology on rabbit eyes treated with Healaflow®. Compounded image of electoretinograms (ERG) before surgery, 1 month after surgery, and 3 months after surgery.
these substances have found their way into routine surgery. Healaflow® is a cross-linked sodium hyaluronic acid hydrogel developed and FDA approved for use in glaucoma surgery [21, 22]. The cross-linking provides the compound with physical properties and structure similar to the collagen and hyaluronic acid meshwork of the natural vitreous [26].

In a recent paper, we described a novel model for in vitro testing of vitreous substitutes, wherein explanted rat retinas kept with Bio-Alcamid®, the PEG-hydrogel, and Healaflow® respectively, were compared to retinas kept with culture medium only. The results were consistent with earlier in vivo trials for the former two substances, which displayed varying degrees of retinal damage, whereas retinas kept with Healaflow® exhibited less culture-induced trauma such as gliosis, loss of structure, and apoptosis compared to those kept with medium only [18]. This suggests excellent biocompatibility, a result confirmed in the current in vivo study. These findings are consistent with other recent animal studies using cross-linked hyaluronic acid hydrogels [17, 31], making this group of substances highly relevant for the development of vitreous substitutes.

In this study, we evaluated Healaflow® for use as a potential vitreous substitute in the rabbit model. The gel was easily incorporated with standard surgical procedures, and well tolerated with no signs of uveitis. Healaflow® was not seen to cause any long-term change in the intraocular pressure, although further studies are required to completely evaluate its effect.

Healaflow® did not affect retinal morphology or function negatively, making it highly interesting in this setting. The gel appeared to maintain its viscous structure in the vitreous cavity for at least a couple of weeks, potentially allowing for an effective short-term tamponade. The fact that the iatrogenic retinal detachments were self-limiting on long-term follow up may support the notion of a tamponading effect, although further studies are clearly warranted to confirm this phenomenon. Future work will include an increased ratio of cross-linking of
the gel to prolong the time of structural integrity even further, potentially allowing for a longer retention time in vivo.

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**Compliance with ethical standards** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

This article does not contain any studies with human participants performed by any of the authors.

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**Conflict of interest** All authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

**References**