A new model for in vitro testing of vitreous substitute candidates

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BASIC SCIENCE

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

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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 perflourocarbon 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 proceedings 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 CO_2 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

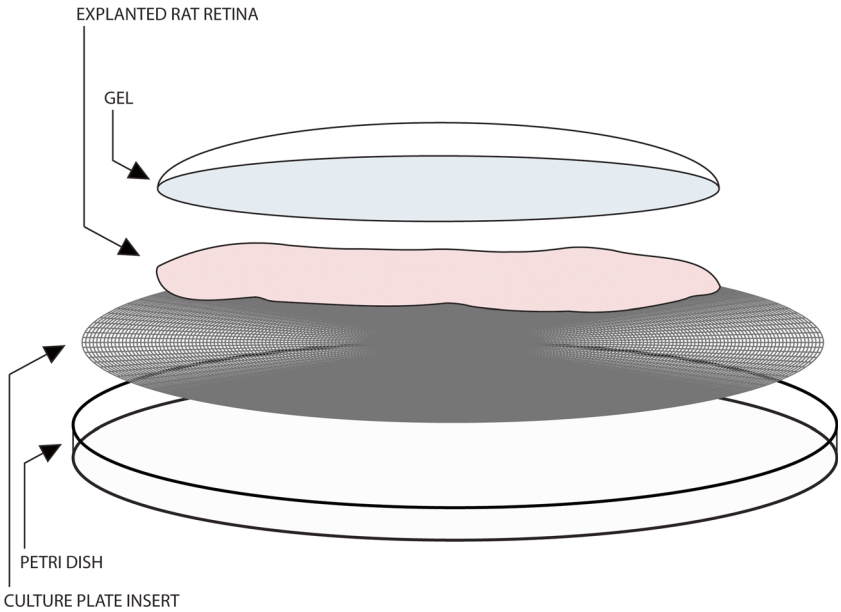


Fig. 1 Schematic overview of the retinal explant culture system with the vitreous substitute candidate

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.

Immunohisto chemistry

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

Table I Specification of immunohistochemical markers

Antigen	Antibody name	Target structure	Species	Dilution	Source
GFAP	Anti-glial fibrillary acidic protein	Astrocytes, activated Müller cells	Mouse monoclonal	1200	Chemicon International, CA, USA
Neurofilament 160 KDa (NF160)	Anti-Neurofilament 160 clone NN18	Ganglion and horizontal cells	Mouse monoclonal	1500	Sigma, St. Louis, MO, USA
PKC	Phospho-PKC (pan)	Rod bipolar cells	Rabbit polyclonal	1200	Cell Signaling, Beverly, MA, USA
Rho4D2	Rod photoreceptor	Rod photoreceptor	Mouse monoclonal	1:100	Kind gift of Prof. RS Molday, Vancouver, Canada
Vimentin	Mouse anti-vimentin	Müller cells	Mouse monoclonal	1:500	Chemicon International, CA, USA
Secondary antibody	**Antibody name**	**Target**	**Species**	**Dilution**	**Source**
FITC	Anti-mouse IgG FITC conjugate	Anti-mouse	Goat	1:200	Sigma, St Louis, MO, USA
FITC	Goat Anti-Rabbit IgM + IgG (H + L chain specific	Anti-rabbit	Goat	1:200	Southern Biotechnology Associates, AL, USA

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.

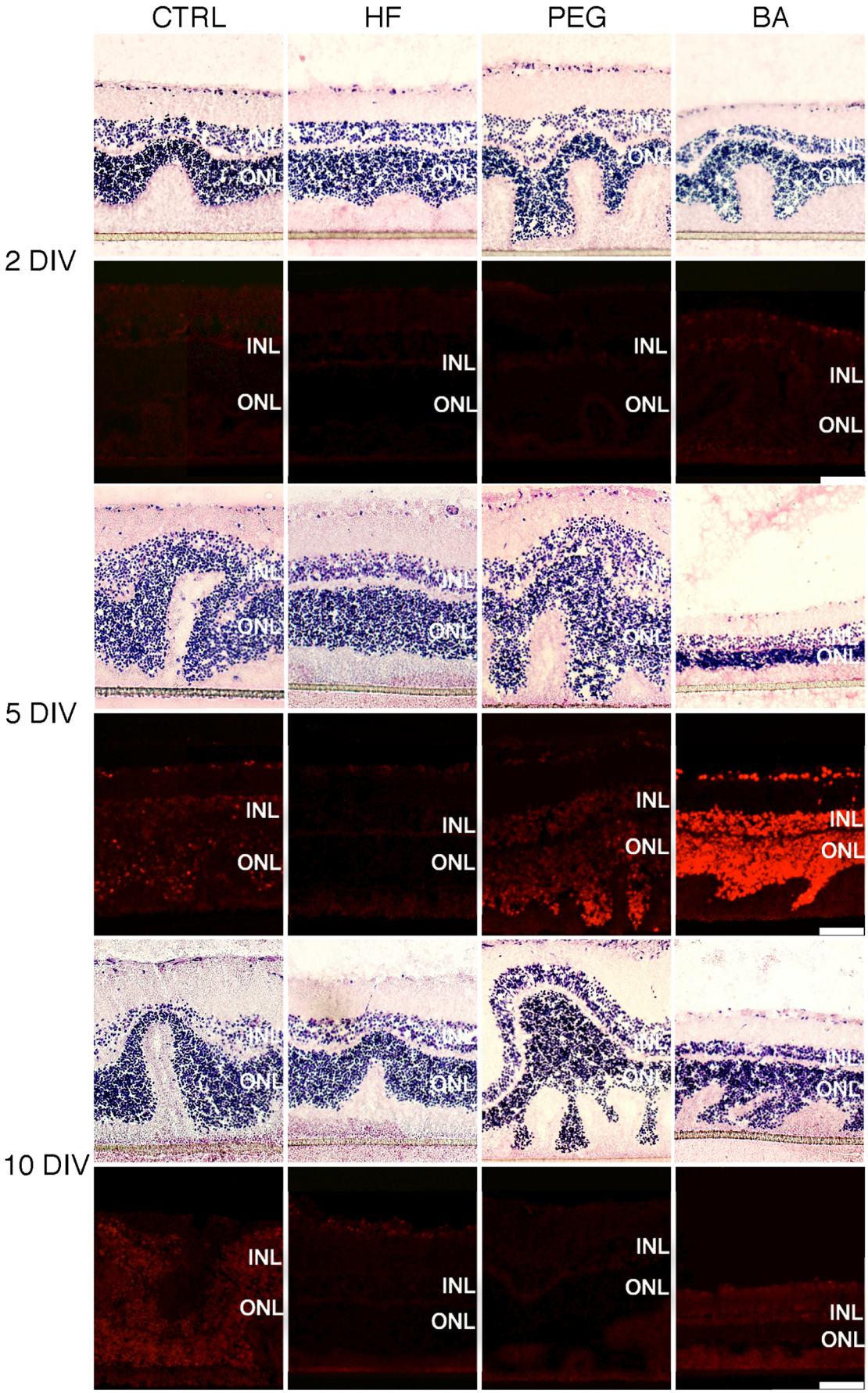
Cytoarchitecture and cell death (Fig. 2)

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

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 ▶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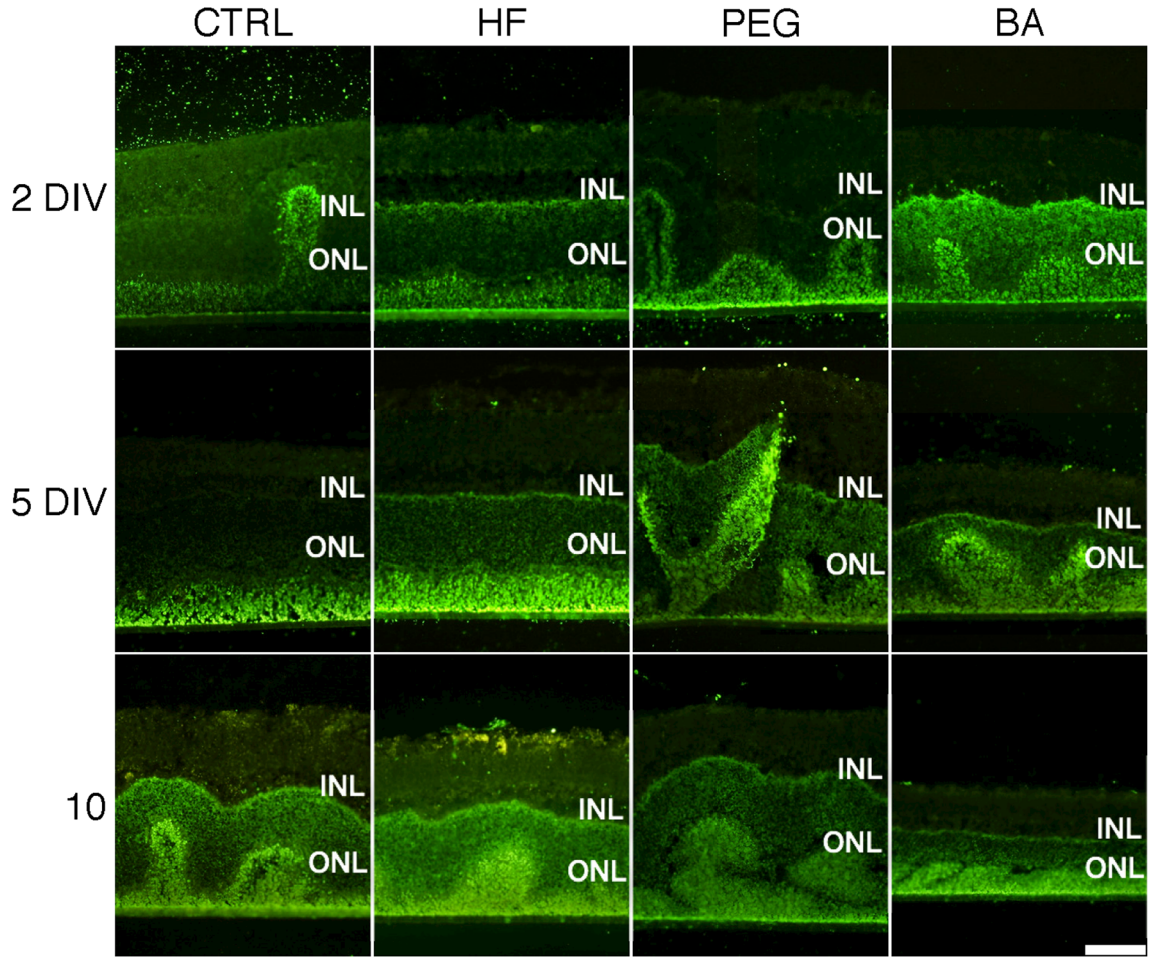


Fig. 3 Cryosections of rat retina explants at 2, 5 or 10 days in vitro (DIV) cultured with: standard conditions (CTRL), Healaflow® (HF), PEG-gel (PEG) and Bio-Alcamid® (BA). Immunohistochemical staining for Rhodopsin. Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=25 μm

control. Bio-Alcamid® explants displayed intense labeling of the entire ONL already at 2 DIV.

Inner retinal cells (Fig. 4)

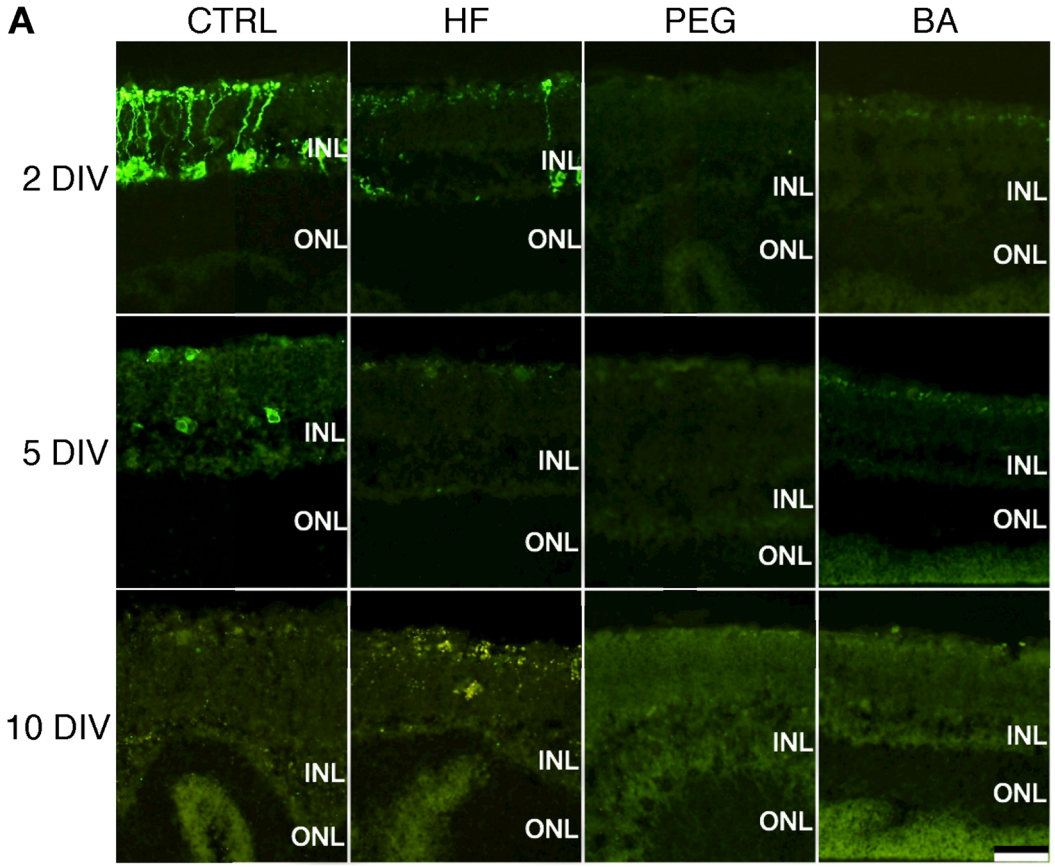
PKC labeling for rod bipolar cells at 2 DIV displayed a high variability with the most intense labeling towards the peripheral edge of the control explants. In 5 DIV specimens only a few PKC-labeled cell bodies were found whereas 10 DIV specimens did not show any remaining rod bipolar cells. In Healaflow®-treated 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

Fig. 4 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). Immunohistochemical staining: **a** PKCpan; **b** Neurofilament 160. Abbreviations: inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar=12.5 μm ▶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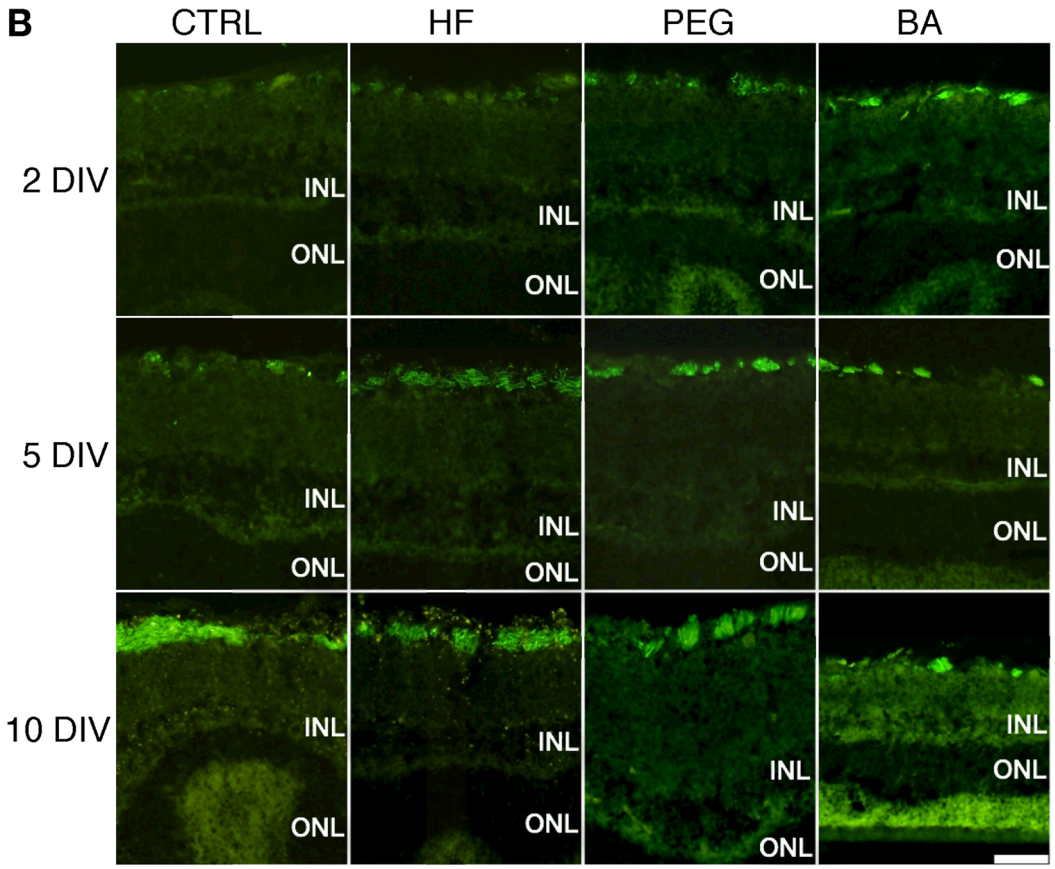
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was a tendency towards a slightly lower labeling intensity at 5 DIV.

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.

Fig. 5 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**). Immunohistochemical staining: **a** *GFAP*; **b** Vimentin. Abbreviations: inner nuclear layer (*INL*), outer nuclear layer (**ONL**). Scale bar=12,5 ▶

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 analysed 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 with minor changes in retinal cytoarchitecture and GFAP-upregulation, and minor electrophysiological changes [38]. On the other hand, 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

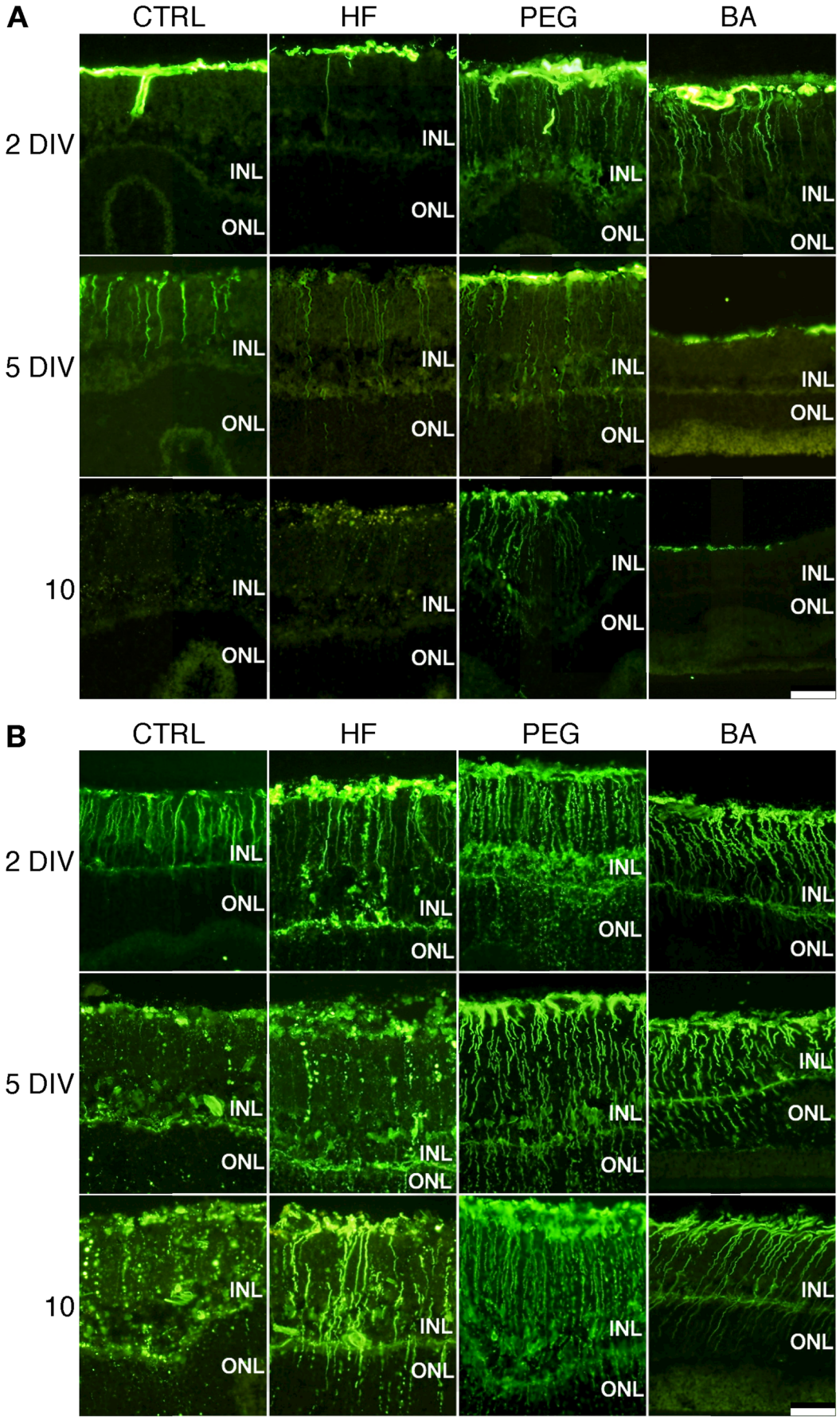
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Table 2 Relative compound score for the degenerative retinal changes for the different gels compared to standard conditions ranging from – to ++

Gels	Cytoarchitecture and cell death	Rod photoreceptors	Inner retinal cells	Müller cell activation
Healaflow®	_	0	+	_
PEG	+	0	++	+
Bio-Alcamid®	++	++	++	++

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 andtime-consumingin 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.

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1986) and the U.S. Animal Welfare Act, as amended, were followed, as well as the current version of the Swedish Law on the Protection of Animals, where applicable.

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