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LABORATORY INVESTIGATION

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Evidence of toxic side effects of perfluorohexyloctane after vitreoretinal surgery as well as in previously established in vitro models with ocular cell types

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Abstract *Background:* Cases of ocular irritation have been observed after early clinical trials using perfluorohexyloctane (F6H8) as endotamponade. In our clinic two of three eyes developed severe inflammatory-like reactions after intermediate-term tamponade. These cases will be depicted, serving as background for the experimental study. To elucidate possible toxic effects of F6H8 on different ocular cell types and corneal tissue we applied our previously established in vitro models to investigate effects of F6H8 on cultured ocular cells in comparison with perfluorodecaline. *Methods:* Vitality and proliferation of cultured human corneal endothelial cells (HCEC) and human retinal pigment epithelial cells (RPE) were measured after incubation with F6H8 or perfluorodecaline for up to 5 days. Vitality was evaluated using the Live/Dead assay, and proliferation was determined according to BrdU incorporation. Additionally the endothelium of donor corneas was incubated with F6H8 for 5 days and endothelial cell morphology was documented. *Results:* After 5 days incubation with F6H8, cultures of RPE and HCEC showed significantly lower extinctions for vital cells as well as a non-significant decrease in proliferation compared with controls. Analysis by means of fluorescence microscopy after treatment with F6H8 or perfluorodecaline revealed decreased cell densities (F6H8 > perfluorodecaline) within contact areas. The endothelium of donor corneas incubated in presence of F6H8 developed circumscribed necrotic areas. *Conclusions:* Decreased amounts of vital cells cannot be explained solely by mechanical effects or nutritional deficit due to direct contact, since F6H8 has a lower specific weight than perfluorodecaline. The ability of the remaining cells to proliferate revealed that they were not irreversibly damaged. Due to the high lipophilicity of F6H8 interactions with cellular lipoprotein membranes as well as other toxic effects have to be considered and should further be investigated prior to clinical use.

Introduction

Liquid perfluorocarbons are established intraoperative tools for manipulation in difficult vitreoretinal surgery [5, 20]. Due to their high specific gravity (1.7–2.1 g/cm^3) hydrokinetic stabilisation of the inferior retina can be achieved. Retinal damage by mechanical effects after prolonged contact with liquid perfluorocarbons is attributed to their relatively high specific gravity [18, 22, 23, 24]. Partially fluorinated alkanes present physical properties comparable to liquid perfluorocarbons [11, 16, 17]. Perfluorohexyloctane ($C_{14}F_{13}H_{17}$; F6H8) is a semifluorinated alkane with lipophilic and hydrophilic properties but a density of only 1.35 g/cm^3. Furthermore, it is a biocompatible solvent for silicon oil [13]. Because of its lower density and its biocompatibility, F6H8 has recently

been used as an intraoperative tool and intermediate-term vitreous tamponade in difficult surgical procedures [25]. Zeana et al. found good intraocular intermediate-term tolerance in a rabbit model for up to 9 weeks. However, in some animals use of F6H8 led to ocular irritation with whitish precipitates and widespread proliferative vitreoretinopathy (PVR) [25]. Hiscott et al. [8] excised membranes after long-term use of F6H8 and detected histological and immunohistochemical features typical for PVR membranes with macrophages and giant cells. Kobuch et al. [12] observed vascular constriction, slight disarrangement of the plexiform layers, hypertrophy of Müller cells and vacuolisation of the inner retinal surface in rabbit eyes after 6 weeks' tamponade.

In our clinic, F6H8 was used as an intermediate-term vitreous tamponade in three patients who suffered from complicated retinal detachment. Two of these patients developed inflammatory-like complications 8–14 weeks after surgery.

Based on this early clinical experience, in vitro tests were performed to determine possible toxic side effects of this new drug applying the Live/Dead assay and a proliferation assay on human retinal pigment epithelium (RPE) and human corneal endothelial cells (HCEC). To assess the effect also in an ocular tissue model in vitro, two pairs of human donor corneas were incubated with F6H8. These tests had previously been established in our lab to elucidate possible toxic effects of perfluorodecaline [18]. Moreover, ocular cell cultures have been shown to be useful in quality testing of media and sera for organ culture [7].

Materials and methods

Vitreoretinal surgery using F6H8

A standard three-port vitrectomy, retinal reattachment and endolaser coagulation was performed in all three patients (patient 1, female, aged 45 years; patient 2, female, aged 75 years; patient 3, male, aged 78 years). F6H8 was used as endotamponade and filled the whole vitreous cavity.

Removal of the tamponade was performed after 6 weeks (patient 3), 8 weeks (patient 1) and 12 weeks (patient 2).

Cell cultures

RPE cells of four different human donors were isolated and cultivated using the method described by Sobottka Ventura et al. [21]. RPE cells were not transfected.

Immortalised HCEC [2] transfected with pRNS [14], a plasmid containing the genes for the SV40 small and large T-antigen, were cultivated in medium F99. a 1:1 mixture of Ham's F12 (Invitrogen) and M199 (Invitrogen), supplemented with 5% fetal calf serum (FCS; Seromed). For subcultivation cells were trypsinised (0.05%/0.02% trypsin/EDTA in phosphate-buffered saline), seeded onto new culture flasks or microtiter plates, and cultivated in an atmosphere containing 5% CO_2 at 37°C.

Live/Dead assay

Vitality of cells was determined using the Live/Dead Viability/Cytotoxicity kit (Molecular Probes) containing the two components calcein and ethidium homodimer. Calcein penetrates the cell membrane, and esterases in the cytoplasm of vital cells render it fluorescent. Ethidium homodimer penetrates not intact but damaged cell membranes and then adheres to nucleic acids of damaged or dead cells, leading to red fluorescent nuclei.

Cell cultures of human RPE and HCEC were seeded on microtiter plates and cultivated until cell layers were semiconfluent. Next, cultures were incubated with an additional 50 µl perfluorodecaline or F6H8 per hole for 1, 3 and 5 days. Controls did not receive these additives. Dying procedure, analysis and documentation were as described before [18].

Proliferation assay

Cultures of RPE and HCEC were treated with F6H8 or perfluorodecaline as described for the Live/Dead assay. After the incubation period, F6H8 or perfluorodecaline was removed by washing the cultures twice with PBS. Subsequently, the cells were cultured for a further 20–24 h in the presence of 10 mM bromodeoxyuridine (BrdU). Incorporation of BrdU was determined by means of monoclonal antibodies as described by Mertens et al. [18].

Organ culture

Two pairs of human corneas not suitable for transplantation due to low endothelial cell density and/ or hepatitis C infection were stored in 50-ml tissue culture flasks. Donor ages were 73 and 80 years, post mortem times 58 and 6 h. Organ culture medium was minimal essential medium supplemented with 2% FCS [2]. In order to determine the effect of F6H8 on corneal endothelium, corneas were preserved in 24-well plates, endothelial side up, in minimal essential medium supplemented with 2% FCS and 6% dextran T500. In each pair, 100 µl F6H8 was applied to the endothelium of one cornea. The fellow corneas were not treated and served as controls. After 1, 3 and 5 days endothelial cell densities were determined using a phase-contrast microscope (Labovert FS, Leitz) and documented photographically [2].

Statistics

Live/Dead assay and proliferation assay were repeated four times using RPE cells from four different donors and four different subcultures of the HCEC cell line. Controls were always performed using cells of the same preparation. The extinction values were normalised to the values obtained with control cultures. The significance of the results was determined using Student's *t*-test.

Results

Two out of three patients developed symptoms of intraocular irritation with white precipitates in the vitreous after intermediate-term endotamponade with F6H8.

Brief case reports

Case 1

Diagnosis. Central retinal detachment due to multiple retinal holes, myopia magna and posterior staphyloma.

Therapy. Standard three-port vitrectomy, F6H8 endotamponade, endolaser treatment.

Follow-up. No intraocular irritation 6 weeks postoperatively; F6H8 removal after 8 weeks.

Complications. Development of retrolental and preretinal grey–white membranes or fibrin-like structures. After F6H8 removal retinal redetachment in four quadrants. Following vitrectomy and silicone oil endotamponade resulted in hypotonia.

Case 2

Diagnosis. Vitreal and subretinal hemorrhage with retinal detachment due to CNV in age related macular degeneration.

Therapy. Standard three-port vitrectomy, lavage of subretinal blood, F6H8 endotamponade, endolaser and endocryocoagulation.

Follow-up. Follow-up examinations not performed due to non-compliance of the patient until 13 weeks after surgery.

Complications. Dispersion and penetration of F6H8 into the anterior chamber (Fig. 1). Development of membrane-like structures similar to case 1, decompensation of intraocular pressure (45 mmHg). Retinal detachment after F6H8 removal.

Case 3

Diagnosis. Amotio non-sanata (inferior quadrants) with risk of macular detachment state post conventional silicone plombage.

Therapy. Standard three-port vitrectomy, F6H8 endotamponade, endolaser coagulation. Because of previous complications F6H8 removal was performed after 6 weeks without inflammatory complications.

Follow-up. The retina remained attached.

Unexpected complications occurred during the postoperative course in two of three patients. Morphological changes in both patients occurred in the anterior vitreous segment related to the posterior surface of the lens. There are grounds for suspicion that interaction of the vitreous tamponade with the retinal surface, persistent anterior vitreous cortex or lens material led to toxic side effects and inflammatory processes. Based on this hypothesis and our clinical observations, in vitro tests were performed using non-transformed cells of ocular origin.

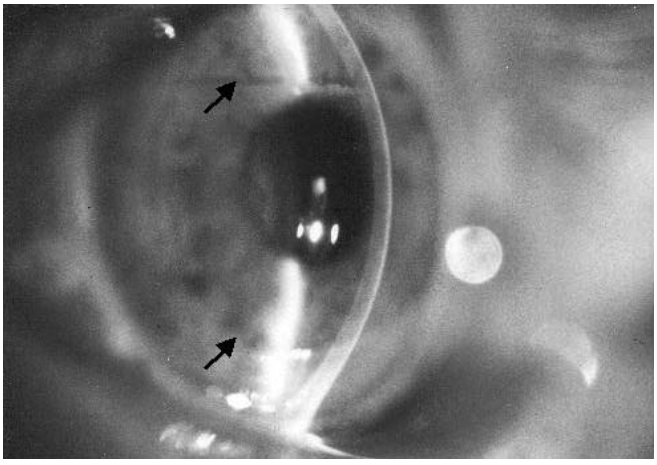

Fig. 1 Anterior segment of the eye of 75-year-old patient E.S. after F6H8 vitreous tamponade for 14 weeks. F6H8 dispersed and penetrated into the anterior chamber (*arrows*)

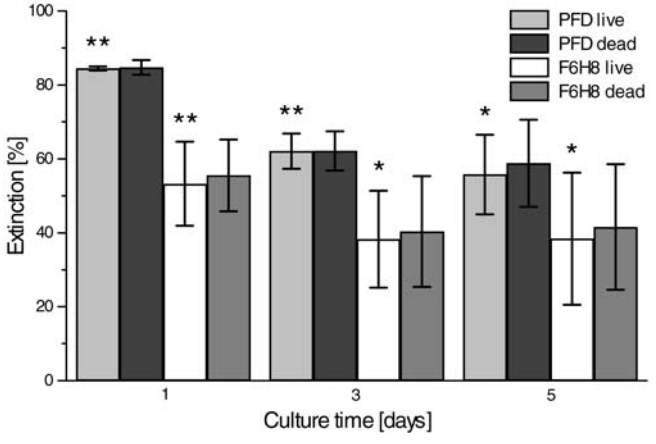

Fig. 2 Effect of perfluorodecaline (*PFD*) and F6H8 on vitality of human RPE. Prolonged contact with PFD and F6H8 caused a progressive decrease of the fluorescent signal emitted by vital or dead cells. Five-day incubation with PFD led to mean extinctions of 56% for vital cells and 59% for dead cells compared with untreated control cultures. F6H8 incubation led to mean extinctions of 38% for vital cells and 41% for dead cells after 5 days. Significance (Student's *t*-test): * $P<0.05$, ** $P<0.01$

Human retinal pigment epithelial cells

Contact of cultured RPE cells with F6H8 and perfluorodecaline led to a reduction in the fluorescent signals emitted by vital and damaged cells respectively. The effect was more evident with F6H8 than after contact with perfluorodecaline. In detail, the values relative to controls for the signals emitted by living cells measured after contact with F6H8 were 53.2%, 38.2% and 38.4% after 1, 3 and 5 days respectively (Fig. 2). The corresponding values after contact with perfluorodecaline were 84.4%, 62.1% and 55.8% (Fig. 2). Thus, the reduc-

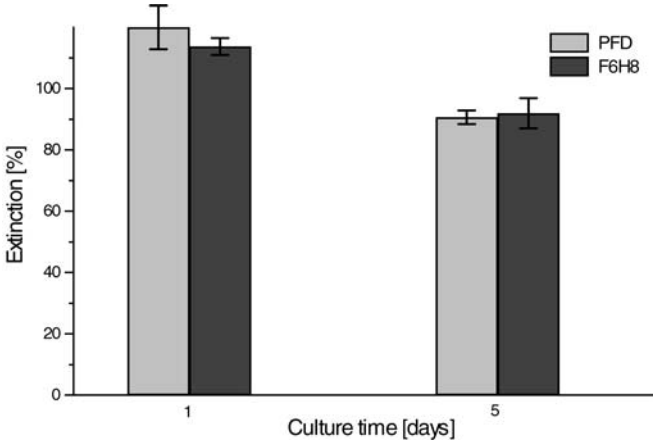

Fig. 3 Mean proliferative activity after incubation of RPE cultures with perfluorodecaline (*PFD*) or F6H8 for up to 5 days. Cell proliferation after contact with the substances was not significantly different from that in controls

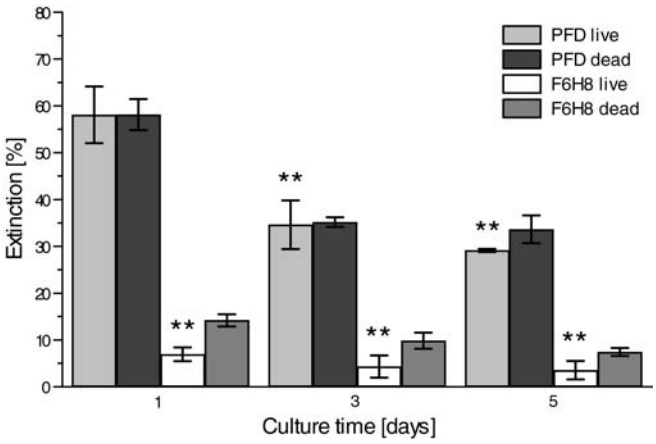

Fig. 4 Effect of perfluorodecaline (*PFD*) and F6H8 on cultured transfected HCEC by means of the Live/Dead assay compared with controls (100%). One-day contact induced a significant decrease in extinctions for vital and dead cells. Significantly reduced values (3–14%) observed after incubation with F6H8 revealed areas of nearly complete cell necrosis. Significance (Student's *t*-test): * $P<0.05$, ** $P<0.01$

tion in fluorescence was higher after contact with F6H8 than after contact with perfluorodecaline at all time points. An analogous result was found for fluorescent signals emitted by damaged cells. After contact with perfluorodecaline the emissions measured at 1, 3, and 5 days reached 84.7%, 62.2%, and 58.8%, respectively, of the values in the control cultures (Fig. 2). Cultures incubated with F6H8 exhibited only 55.5%, 40.3% and 41.6% of the fluorescent signal emitted by untreated cell cultures (Fig. 2).

Although contact of cell cultures with F6H8 or perfluorodecaline caused a decrease in fluorescent signals characteristic for living as well as for damaged cells, the relation between these two signals remained rather constant during the observation period.

After removal of the substances from cultures, the remaining proliferative capacity was determined and compared with controls. No significant changes in proliferative activity after incubation with either substance were observed. Extinctions of 90.6% (*t*-test: $P=0.019$) after 5-day contact with perfluorodecaline and 91.9% (*t*-test: $P=0.104$) with F6H8 were measured (Fig. 3).

Human corneal endothelial cells

Cultured HCEC responded to contact with F6H8 or perfluorodecaline in the same manner as RPE cells but even more sensitively. As shown in Fig. 4, after only 1 day's contact with F6H8 the signal emitted by living cells had decreased to only 6.9% of that in untreated cells. Further contact caused an ongoing decrease to 4.3% after 3 days and 3.5% after 5 days. At the same points in time, the signal emitted by damaged cells reached only 14.2%, 9.8% and 7.4%, respectively, of that emitted by the controls (Fig. 4). The effect was less evident after contact with perfluorodecaline, as already seen in cultured RPE cells. The emission from vital cells was 58.1%, 34.6% and 29.1% of the control values at 1 day, 3 days and 5 days, respectively, and that from damaged cells was 58.1%, 35.2% and 33.6%.

The decrease in emission characteristic for vital cells was not correlated with an increase in the fluorescent signal emitted by damaged cells. Analysis of the stained cell cultures by means of fluorescence microscopy revealed that after treatment with F6H8 or perfluorodecaline a decrease in cell density occurred within the regions of F6H8 or perfluorodecaline application (Fig. 5). Therefore the overall decrease in fluorescence corresponded to an overall decrease in cell number.

The cells remaining after incubation in the presence of F6H8 and perfluorodecaline were analysed for their proliferation activity as indicated by incorporation of BrdU. Contact with perfluorodecaline did not affect BrdU incorporation into HCEC significantly. After contact with F6H8 a decrease in proliferation activity compared with untreated cells was observed, but the difference was not statistically significant ($P=0.072$).

Organ-cultured corneas

The endothelium of cultured human corneas did not show a significant decrease in cell density after incubation with F6H8 compared with controls. Circumscribed areas of cell necrosis developed at the interface between medium and F6H8 after 3 days. Since only two pairs of human corneas were incubated with F6H8 no statistical evaluation of mean cell density was attempted.

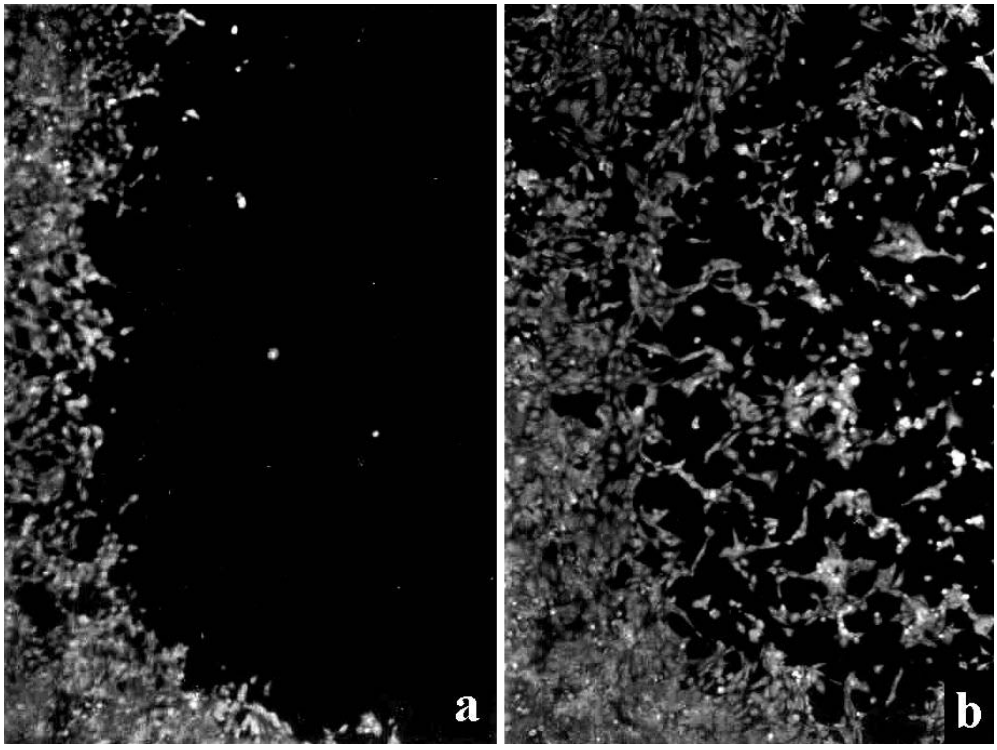

Fig. 5a, b Cultured HCEC after incubation with **a** F6H8 and **b** perfluorodecaline for 3 days. Cultures were analysed using the Live/Dead assay. In areas which had been in contact with F6H8 or perfluorodecaline cell density was dramatically decreased

Discussion

Retinal complications after F6H8 endotamponade in two of three eyes were unexpected and led to severe decrease in visual acuity. Removal of the newly formed membrane-like structures which had developed between the inner retinal layer and F6H8 tamponade proved difficult. The material was extremely fragile and could not be peeled off like a membrane. Structures were especially situated in the anterior part at the vitreous basis and retrolental. Despite the absence of signs of inflammation in the anterior segment, findings were comparable to endophthalmitis-like reactions. Interaction between F6H8 and remnants of the vitreous cortex in phacic eyes has to be considered. The origin of precipitates and membranes especially at interfaces, e.g. lens capsule, anterior vitreous and between retina and endotamponade, remain to be elucidated. Observation of these clinical cases led us to perform an in vitro trial to analyse possible toxic effects of F6H8.

F6H8 is licensed for use in complicated retinal detachments especially in the inferior part of the eye. In Germany animal studies [25] and standard toxicity tests were performed prior to clinical use. Toxicity tests were performed using HeLa, MonoMac 6, Molt 4 and Raji cell cultures [17]. These cells have been under culture conditions for many decades and have lost some metabolic features of the original cell types. They are mainly used for toxicity testing to evaluate effects on the housekeeping metabolism of cells [15].

In our study we used cultures of human ocular cells which may come into contact with F6H8 after its use as endotamponade. Both cell types, primary cultured RPE [21] and transfected HCEC [1, 3], retained morphological and functional characteristics during culture and have been used successfully in previous test systems to evaluate toxic effects [2, 7, 18]. Furthermore, we brought these cultured cells in direct contact to F6H8. Therefore we believe that this model reflects the clinical situation after endotamponade more accurately than other in vitro test systems used before. In cultures of both cell types, contact with F6H8 led to a decrease in the number of vital cells. Only cells which had been in contact with F6H8 seemed to be damaged, however. After removal of this substance, the remaining cells of the cultures exhibited normal proliferation activity. Possible mechanical and barrier effects of the substances during incubation have to be considered since cells were seeded onto the floor of culture plates [18]. The reasons for the significant effect of F6H8 remain unclear, but our results yield some evidence of interaction between F6H8 and cellular structures.

Ten years ago low- molecular-weight components were shown to be responsible for toxic effects seen after silicone oil application [19]. In the case of F6H8 the observed effects cannot be explained by such contaminations since these preparations are stated to be 100% pure.

The effect of cell loss was much more pronounced after F6H8 application than after use of perfluorodecaline. The hydrogenation of F6H8 causes a polarisation

and renders this substance more lipophilic [10]. Thus F6H8 may interfere with the cell membrane, membrane proteins or other hydrophobic proteins such as albumin. Interaction with cell membranes may change the adherence of the cells to their matrix. This would explain the occurrence of cell-free areas in cultures treated with F6H8. In contrast, the endothelium of donor corneas is additionally stabilised by tight junctions between the endothelial cells. These tight junctions are not yet present in non-confluent cell cultures. Therefore, cultured cells may be more susceptible to F6H8-associated damage. Nevertheless, necrosis developed also in the endothelium of cultured organ corneas at the interphase between F6H8 and medium. Thus, F6H8 seemed to exert its effects not only by influencing the adherence of the cells to their matrix. Compared with an in vivo model, cell cultures do not present vascular structures. Therefore, observation of protein leakage and macrophages described histologically as whitish membranes and PVR formation cannot be observed using this in vitro model.

Whitish precipitates observed in patient eyes may be blood derived or of cellular origin. Hoerauf and Laqua [9] described emulsification after combined use of silicone oil and perfluorohexylhexane, a semifluorinated alkane, for macular rotation. Besides emulsification and opacification, proteins as well as lipids, blood cells, fibrin, albumin and fibrinogen have been found after endotamponade in a human eye. A breakdown of the blood–retina barrier has to be expected in this case. Kobuch et al. [12] described alteration of vascular structures 6 weeks after injection of a semifluorinated alkane (O62) into the rabbit eye. Vasoconstriction may cause disturbance of the blood–retina barrier, resulting in leakage of protein. This would explain the presence of albumin demonstrated in the whitish precipitates seen after the use of hydrofluorocarbon liquids (Spindler, unpublished communication, DOG 2001) as well as the occurrence of PVR-like membranes with macrophages and giant cells [8]. Why these reactions have been observed after different times of incubation remains unclear.

Additional in vitro tissue tests and animal studies should be considered prior to further clinical application of F6H8 in patients with retinal detachment.

References

1. Aboalchamat B, Engelmann K, Böhnke M, Bednarz J (1999) Morphological and functional analysis of SV40 transfected human corneal endothelial cells after transplantation. Exp Eye Res 69:547–552
2. Bednarz J, Aboalchamat B, Engelmann K (2000) Characterization of immortalized human corneal endothelial cells [abstract]. Invest Ophthalmol Vis Sci 41:448
3. Bednarz J, Teifel M, Friedel P, Engelmann K (2000) Immortalization of human corneal endothelial cells using electroporation protocol optimised for human corneal endothelial and human retinal pigment epithelial cells. Acta Ophthalmol Scan 78:130–136
4. Böhnke M (1991) Corneal preservation in organ culture. Curr Opin Ophthalmol 2:432–442
5. Chang S, Zimmerman HJ, Iwamoto T (1997) Experimental vitreous replacement with perfluortributylamine. Am J Ophthalmol 103:29–37
6. Engelmann K, Friedl P (1989) Optimization of culture conditions for human corneal endothelial cells. In Vitro Cell Dev Biol 25:1065–1072
7. Engelmann K, Sobottka-Ventura A, Drexler D, Staude HJ (1998) A sensitive method for testing the quality of organ cultured media of individual medium components in a cornea bank. Graefes Arch Clin Exp Ophthalmol 236:312–319
8. Hiscott P, Colthurst, Wong D, Lois N (2001) Epiretinal membranes and long-term perfluorohexyloctane tamponade. ARVO Abstract 2305
9. Hoerauf H, Laqua H (2002) Severe emulsification after combined use of a partially fluorinated alkane and silicone oil. Graefes Arch Clin Exp Ophthalmol 240:131–136
10. Hoerauf H, Kobuch K, Dresp J, Menz D (2001) Combined use of partially fluorinated alkanes, perfluorocarbon liquids and silicone oil: an experimental study. Graefes Arch Clin Exp Ophthalmol 239:373–381
11. Kirchhof B (1999) Fluorkarbone in der Netzhaut-Glaskörperchirurgie. Ophthalmochirurgie 11:153–158
12. Kobuch K, Menz DH, Hoerauf H, Dresp JH, Gabel VP (2001) New substances for intraocular tamponades: perfluorocarbon liquids, hydrofluorocarbon liquids and hydrofluorocarbon-oligomers in vitreoretinal surgery. Graefes Arch Clin Exp Ophthalmol 239:635–642
13. Langefeld S, Schrage N, Meinert H, Roy T, Aretz A, Kirchhof B (1999) A new way of removing silicone oil from the surface of silicone intraocular lenses. Graefes Arch Clin Exp Ophthalmol 237:201–206
14. Litzkas P, Jha KK, Ozer HL (1984) Efficient transfer of cloned DNA into human diploid cells: protoplast fusion in suspension. Mol Cell Biol 4:2549–2552
15. Marquardt H, Schäfer G (1994) Lehrbuch der Toxikologie, vol I. Wissenschaftsverlag, Mannheim, p 138
16. Meinert H, Geister U (1992) Semifluorinated compounds in ophthalmology. J Vitreoretina 1:31–35
17. Meinert H, Roy T (2000) Semifluorinated alkanes – a new class of compounds with outstanding properties for use in ophthalmology. Eur J Ophthalmol 10:189–197
18. Mertens S, Bednarz J, Richard G, Engelmann K (2000) Effect of perfluorodecaline on human retinal pigment epithelium and human corneal endothelium in vitro. Graefes Arch Clin Exp Ophthalmol 238:181–185
19. Nakamura K, Refojo MF, Crabtree DV, Pastor J, Leong FL (1991) Ocular toxicity of low-molecular-weight components of silicone and fluorosilicone oils. Invest Ophthalmol Vis Sci 32:3007–3020
20. Peyman G, Schulman J, Sullivan B (1995) Perfluorocarbon liquids in ophthalmology. Surv Ophthalmol 39:375–395
21. Sobottka Ventura A, Böhnke M, Löliger C, Kühnl P, Winter R, Engelmann K (1996) HLA-Typisierung von Spenderhornhäuten mit hohen post-mortem-Zeiten. Ophthalmologe 93:262–267

22. Stolba U (1996) Perfluorocarbone als Glaskörperersatz – klinische und experimentelle Ergebnisse. In: Faulborn J, Freyler H, Göttinger W (eds) Spectrum Augenheilkunde 10 Suppl 19. Springer, Berlin Heidelberg New York, pp 3–28
23. Wilbanks G, Apel A, Jolly S, Devenyi R, Rootman D (1996) Perfluorodecaline corneal toxicity: five case reports. Cornea 15:329–334
24. Winter M, Beehrende S, Menz DH, Pfister G (1999) Retinale Druckbelastung durch Perfluordecalin. Ophthalmologe 1 [Suppl]:103
25. Zeana D, Becker J, Kuckelhorn, Kirchhof B (1999) Perfluorhexyloctane as a long-term vitreous tamponade in the experimental animal. Int Ophthalmol 23:17–24