Universitat Autònoma de Barcelona

COLORED PERFLUOROCARBON LIQUIDS IN VITREORETINAL SURGERY IN THE PIG EYE

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PhD Thesis

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Certifies,

That **Fábio Miguel Vasconcelos Trindade**, MD, has worked under my direction in the course of his research project entitled:

"Colored Perfluorocarbon Liquids in Vitreoretinal Surgery in the Pig Eye"

Having reviewed and supervised personally the project I consider that it meets the conditions required to be defended as a PhD thesis.

And for the record, I sign this:

Barcelona, 4th of September of 2013

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José García-Arumí

Acknowledgements

To the most important people in my life:

Leyre Trindade, my daughter.

Elisabet Muñoz, my wife.

Carlos Trindade and **Lídia Trindade**, my parents.

Octávio Trindade and **João Pedro Trindade**, my brothers.

Special mention to those without which this work would not be possible:

Dr. Jose García-Arumí, Opthalmologist-in-chief and PhD Director, for his support, friendship and constant availability, but also for its thoroughness, scientific method and encouragement. From his example, all I have learned is not quantifiable.

Mr. Mauro Beccaro, AL.CHI.MI.A, CEO, for the invaluable discussion and support during all experiments.

To all people who participated in my training as a resident in Coimbra (PT), especially to:

Dr. Roque Loureiro, CHC, Ophthalmologist-in-chief; **Dr. João Filipe**, CHC, Glaucoma Specialist; **Dr. Filipe Rito** and **Dr. Vivelindo Branco**, CHC, Cataract and Refractive Surgeons; **Dr. Augusto Barbosa** and **Dr. Rui Pinheiro**, CHC, Retinal Specialists; **Dr. Rui Castela**, CHC, Pediatric Ophthalmologist and Strabismus Specialist; **Dr. João Matias**, CHC, older residence colleague and friend; and **Mr. Marcelo Seara**, CHC, Orthoptist.

To all people who participated in my retina fellowship in Barcelona (ES), especially to:

Dr. Borja Corcóstegui, IMO, Ophthalmologist-in-Chief; **Dr. Jose García-Arumí** and **Dr. Carlos Mateo**, IMO, Surgical Retina Specialists; **Dr. Alfredo Adán**, IMO, Uveitis Specialist and **Dr. Rafael Navarro**, IMO, Medical Retina Specialist.

To all those people that actively participated during some stages of the study:

Mr. Rafael Martin, IMO, Surgical Technician Coordinator; **Ms. Elisabet Muñoz**, IMO, Surgical Nurse; **Mr. Carmelo Jaén**, IMO, Surgical Technician, **Mr. Jesus Mures**, IMO, Surgical Technician; and **Mr. Alejandro Couto**, IMO, Audio Visual Technician, for the support during the study period and in particular the surgical procedures of the “ex vivo” stage of the study.

Ms. Andrea Carvalho, VHIR, Veterinarian Opthalmologist; **Ms. Anna Salas**, VHIR, Biochemist; **Dr. Miguel Zapata**, Vall D’Hebron Hospital, Ophthalmologist; and **Dr. Laura Distefano**, Vall D’Hebron Hospital, Ophthalmologist, for the support during the “in vivo” stage of the study.

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Glossary of Abbreviations

Abbreviation	Term	Abbreviation	Term
AMD	Age-related Macular Degeneration	MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
ARVO	Association for Research in Vision and Ophthalmology	N_2	Nitrogen Gas
BSS	Balanced Salt Solution	NMR	Nuclear Magnetic Resonance
C_3F_8	Perfluoropropane	ONL	Outer Nuclear Layer
CHC	Centro Hospitalar de Coimbra	OPL	Outer Plexiform Layer
CNV	Choroidal Neovascularization	PEEP	Positive End-Expiratory Pressure
DNA	Deoxyribonucleic acid	PIPmax	Maximum Peak Inspiratory Pressure
ERG	Electroretinogram	PFCL	Perfluorocarbon Liquid
F_6H_8	Perfluorohexyloctane	PFD	Perfluorodecalin
FDA	Food and Drug Administration	PFnO	Perfluoro-n-Octane
Fig	Figure	PFPHP	Perfluoroperhydrophenanthrene
G	Gauge	PRL	Photoreceptor Layer
GCL	Ganglion Cell Layer	PTFE	Polytetrafluoroethylene
IMO	Instituto de Microcirurgia Ocular	PVR	Proliferative Vitreoretinopathy
INL	Inner Nuclear Layer	RGC	Retinal Ganglion Cell
IPL	Inner Plexiform Layer	RNFL	Retinal Nerve Fiber Layer
ISCEV	International Society for Clinical Electrophysiology of Vision	RPE	Retinal Pigment Epithelium
ISO	International Organization for Standardization	rTPA	recombinant Tissue Plasminogen Activator

Abbreviation	Term	Abbreviation	Term
SFA	Semifluorinated Alkane	VEGF	Vascular Endothelial Growth Factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling	VHIR	Vall d'Hebron Research Institute

1. INTRODUCTION

1. INTRODUCTION

1.1 History

Perfluorocarbon liquids (PFCLs) are inert liquids that possess an extensive capacity for oxygen transportation. Due to these properties they where first developed as blood substitutes.

In 1966, Clark[1] experimented with liquid breathing in a landmark experiment. He demonstrated that mice immersed for one hour in an oxygenated perfluorocarbon could survive for several weeks after removal from this liquid (Fig. 1). The mice he used later died due to trauma to their lungs, although it could have been due to impurities in the perfluorocarbon.

Fig. 1 Mouse immersed in an oxygenated perfluorocarbon liquid.

The first experiment evaluating the use of PFCLs as a vitreous substitute was presented in 1982 at the Association for Research in Vision and Ophthalmology (ARVO) by Haidt et al[2] and later Clark[3] advocated their use as an intra-operative tool and as a post-operative vitreous substitute.

This was expanded upon by Zimmerman and Ferris[4] in 1984 who reported the use of the PFCLs as intra-operative tools to reposition experimentally detached retinas. After producing breaks and consequent detachment in the inferior retina in rabbits, they injected perfluoroamines, which held the retina in place, and therefore concluded that PFCLs were useful in the management of retinal detachments with inferior breaks.

The first study to use PFCLs in humans was reported by Chang[5]. He described the use of perfluorotributylamine and perfluorodecalin during vitreous surgery of four patients with complicated retinal detachment. Two patients had severe proliferative vitreoretinopathy and the other two had giant retinal tears. The PFCLs

allowed intra-operative flattening of the retina without turning the patient into the prone position and allowed adequate tamponade that avoided a posterior retinotomy for internal drainage of subretinal fluid, respectively.

Recently due to their physical and chemical properties, PFCLs have been used in a myriad of ophthalmic surgical procedures.

1.2 Physical and Chemical Properties

The PFCLs are organofluorine compounds that contain strong carbon-fluorine bonds. The straight chains from C_5 to C_9 are liquids, as are most of the cyclic compounds in the C_5 to C_{17} range. The chemical and physical properties that have propelled their use in vitreoretinal surgery include high specific gravity, high interfacial tension, optic clarity, low viscosity and immiscibility with water, balanced salt solution (BSS), blood or silicone oil. Table 1 describes the physical properties of some PFCLs that have been typically used in vitreoretinal surgery.

High Specific Gravity: The Specific Gravity of PFCLs is between 1.7 and 2.1, and being greater than that of water [sp 1.0], allows for hydrokinetic manipulation of a detached retina, gently tamponading the retina against the underlying retinal pigment epithelium (RPE). This causes the subretinal fluid to exit through the anterior retinal breaks and obviating the need for a posterior drainage retinotomy. It also allows the stabilization of the posterior retina facilitating the removal of anteriorly located membranes and diminishing the risk of iatrogenic damage from the vitrectomy probe.[6]

High Interfacial Tension: Decreases the risk of subretinal migration of the PFCLs which are cohesive substances that tend to remain in one large bubble.

Optic Clarity: Allows for intraocular laser photocoagulation to the attached retina, ensuring adequate postoperative retinopexy. Epiretinal and internal limiting membrane peeling may also be performed under PFCLs.[7,8] These liquids also have refractive indexes slightly different from saline, allowing the use of conventional contact or non contact lenses during surgery. The greater the

difference of refractive index from water the more visible will be the interface between the PFCL and the BSS.

Low Viscosity: PFCLs are less viscous than silicone oil and some less viscous than water. Their viscosities range from 0.8 to 8.0 cSt at 25ºC. This facilitates injection and aspiration through small gauge instruments and tissue manipulation.

Immiscibility: PFCLs are immiscible with water, blood and silicone oil. This property improves visibility when encountering intraocular hemorrhage or performing silicone oil-PFCL exchange.

There are also two other important characteristics to take into account when analyzing the physicochemical characteristics of PFCLs. Firstly, they have a **boiling point** higher than that of water, which allows the application of endophotocoagulation without the risk of vaporization of the PFCL. Secondly, their higher **vapor pressure** is leveraged by some researchers to use PFCLs due to the higher higher capacity they have to vaporize when in contact with air.[9] This would allow small droplets of PFCL remaining on the retina to vaporize under an air exchange.

Table 1. Physical Properties of Some Perfluorocarbon Liquids

Compound	Perfluoro-n-octane (PFnO)	Perfluorodecalin (PFD)	Perfluoroperhydro-phenanthrene (PFPHP)
Chemical Formula	C_8F_{18}	$C_{10}F_{18}$	$C_{14}F_{24}$
Molecular Weight	438	462	624
Specific Gravity	1.76	1.94	2.03
Surface Tension (dyne/cm at 25ºC)	14	16	16
Refractive Index	1.27	1.31	1.33
Vapor Pressure (mmHg at 37ºC)	50	13.5	< 1
Viscosity (cSt at 25ºC)	0.8	2.7	8.03

1.3 Clinical Indications for Use

The PFCLs were firstly described as an indication for complex retinal detachments and with further knowledge and time their indications were expanded.

PFCLs fill the eye in a posterior-to-anterior direction, tamponading the retina against the underlying retinal pigment epithelium (RPE) and displacing the subretinal fluids through the anterior breaks into the vitreous cavity or blood out of the macular area, avoiding a posterior retinotomy.

The greater-than-water specific gravity, immiscibility and low viscosity allow for a relatively atraumatic tissue manipulation during vitrectomy, acting as a “third hand” during membrane peeling.

They also allow for protection of the macular area during manipulation of luxated intraocular and crystalline lenses or intraocular foreign bodies.

A brief discussion of all clinical indications and PFCLs utility will be described bellow.

1.3.1 Retinal Detachments

1.3.1.1 Without Proliferative Vitreoretinopathy

It is generally recognized that in the last 10 years the preferred technique for treating these cases is shifting from scleral surgery to vitrectomy with or without the association of an encircling band or a buckle. This is due to several reasons primarily the presence of an occult break. PFCLs **can help to identify the location of an occult break(s)** by displacing the subretinal fluid through the peripheral break and allowing the observation of *schlieren*, seen when light passes through incompletely mixed fluids having different refractive indices.[10] Another technique would involve injecting trypan blue on the subretinal space and extrusion through the unidentified breaks using a PFCL. This is referred to as Dye extrusion technique (DE-TECH).[11]

Another advantage of using PFCLs in these retinal detachment scenarios could be the potential for decreasing the incidence of full-thickness[12,13] and partial-thickness retinal folds.[14] Retinal folds are rare and likely under reported as they are a potentially severe complication following retinal detachment surgery and are probably due to incomplete internal or external drainage. When vitrectomy is the preferred technique to repair the retinal detachment, PFCLs can be used to promote a more complete internal drainage and in this way **reduce the occurrence of both full-thickness and partial-thickness retinal folds**, especially after a recent onset retinal detachment running through the fovea (Fig. 2). By allowing anterior displacement of the subretinal fluid PFCLs **obviate the need for a posterior drainage retinotomy**. This reduces retinal damage and consequent scotoma, and also decreases the risk of proliferation from this posterior iatrogenic break.

Fig. 2 Recent onset macula-off retinal detachment running through the fovea. This type of retinal detachment has a bigger risk of developing a macular fold on the postoperative period if insufficient attention is given to the internal or external drainage of the subretinal fluid when gas is used as a tamponading agent.

1.3.1.2 With Proliferative Vitreoretinopathy

The use of PFCLs has significantly changed the management of retinal detachments arising from proliferative vitreoretinopathy (PVR).[6,9] They allowed **improvement of anatomic and functional results**, with re-attachment rates ranging from 84% to 96%, and final visual acuities of 20/400 or better in 74% of patients.[15,16]

The PFCLs also **reduced the retinal trauma during surgical manipulation** by stabilizing the macula and allowing a safer, quicker and improved removal of posterior and anterior membranes, thus reducing the duration of surgery.[17]

With the use of PFCLs, a posterior to anterior approach to PVR can be made. Evident subretinal membranes that produce traction should be dissected through the retinal tear or a retinotomy, and epiretinal membranes should be dissected from the macula and optic disc to the periphery. After initial dissection, a small amount of PFCL can be injected close to the optic disc, avoiding the macular area. As membrane dissection proceeds anteriorly, immobilization of the posterior retina with the PFCL reduces the chance of iatrogenic injury.[18] A bi-manual dissection technique can also be used to further reduce the development of iatrogenic breaks.[16]

When adequate membrane dissection has been made, PFCLs will **open the funnel detachment** and will either flatten the retina or **expose any residual areas of traction** that need to be addressed.[6,18,19,20] Aside from flattening and immobilizing the posterior retina, PFCL can **protect the posterior retina from cells and biochemically active substances** produced during surgery which may contribute to reproliferation.[18] If present the mechanical force of PFCLs can be used to **remove an attached posterior hyaloid**.[21]

In eyes with anterior PVR, the PFCLs help to visualize membranes by **opening peripheral folds** while pulling the anterior retina posteriorly. In cases of foreshortening where persistent retinal traction cannot be removed at the vitreous base, peripheral relaxing retinotomies can be performed anterior to the level of the

PFCL.[22,23,24] By containing the posterior retina in situ, PFCLs **eliminate the need for retinal tacks**[25,26,27,28] to fixate the retina after the retinotomy is performed.

In contrast with air, PFCLs **do not cause pupillary miosis** when injected into the eye to flatten the retina.[29]

PFCL-perfused vitrectomy[30] and later en-bloc perfluorodissection[31] have been described for the treatment of proliferative diabetic retinopathy and rhegmatogenous retinal detachment with PVR. Subretinal perfluorocarbon liquid for dissection of PVR has also been recently described.[32] Although some advantages may arise from each technique, prospective and comparative studies are necessary to establish formal indications and possible contra-indications of these techniques.

1.3.1.3 Secondary to Giant Retinal Tears

The introduction of PFCLs in vitreoretinal surgery offered great advantages in the management of giant retinal tears.[33] Techniques that pre-dated the use of these heavy liquids included prone positioning of the patient during fluid-air exchange,[34] use of silicone[35] and fluorosilicone oil[36], and sodium hyaluronate 1%.[37] These techniques have been essentially abandoned due to the difficulty and related complications with these maneuvers, which were often unsuccessful.

The PFCLs allow to **unfold the retinal flap with the patient in the usual supine position** prior to fluid-gas exchange.[38] If an extensive slippage of the tear occurs during air-fluid exchange, then PFCL should be re-injected after air is replaced by saline. A direct silicone oil-PFCL exchange could prevent the slippage phenomena as a result of the mechanical advantage of oil over gas.

After the retinal flap is in its proper position, additional PFCL is added above the level of the tear, and endophotocoagulation can be applied through the PFCL interface along the posterior edge of the giant retinal tear.[39]

Management strategies involving PFCLs allowed for re-attachment rates of more than 90%.[38] The major advantages of this approach include **gentle manipulation**

of the tear and ability to perform **retinopexy with the retinal flap unfolded in its original position**.

The PFCLs may allow to treat certain cases of giant retinal tears without the need for a scleral buckle.[40] In these cases special attention should be paid to the shaving of the vitreous base and endophotocoagulation should be applied to the edges of the tear. However photocoagulation of the peripheral retina over 360 degrees appears to decrease the risk of secondary peripheral retinal tears.[41]

1.3.2 Ocular Trauma

The PFCLs can help in the management of retinal detachments which are secondary to ocular trauma,[7,42] often complicated with lens opacification, vitreous hemorrhage and subretinal or choroidal hemorrhage.

After creating a hole on the posterior hyaloid (if present) PFCLs should be injected in the retrohyaloid space. This will help to **stabilize the detached retina**, obviating the need for a posterior retinotomy, and **dissect the posterior hyaloid** while **anteriorly displacing any vitreous hemorrhage**.[42] Subretinal fluid or hemorrhage are also displaced anteriorly and can be aspirated through the peripheral retinal break.

PFCLs can **simplify the management of organic foreign bodies**, by making them float anteriorly on their surface.[43] Metallic foreign bodies, although heavier than PFCLs, may be easier to grasp once fixated by the PFCL.[44]

Traumatic retinal incarceration may result from severe blunt or penetrating ocular trauma. Traction from the incarceration site may result in complicated retinal detachments which are difficult to repair. PFCLs may assist in the management of this situation.[45,46] In some cases the placement of an explant over the area of incarceration may help to reduce retinal traction. In others, a relaxing retinotomy may be required to relieve traction. In these cases PFCLs can **help to determine the extent of the retinotomy required for adequate retinal relaxation**.[47]

1.3.3 Dislocated Intraocular and Crystalline Lenses

The use of PFCLs has promoted the **safe removal of posterior dislocated intraocular or crystalline lens**,[48] **and nucleus or nuclear fragments**.[49] They are particularly useful in cases with sub-optimal visualization.[50]

Vitrectomy should be done removing all vitreous adhesions to the lens. PFCLs are then introduced to **allow the lens to float off the retinal surface** into the anterior vitreous cavity with minimal trauma, avoiding damage to the retina from posterior falling particles. In the presence of a tear, the PFCL **prevents the retina from detaching**,[51] and in the presence of a retinal detachment it **prevents migration of lens material into the subretinal space** by closing off the retinal breaks.[52,53]

The PFCL interface should be at the equator to eliminate the possibility of entrapment of small nuclear fragments in residual vitreous.[51] Crystalline lens or nuclear fragments can then be removed with the fragmatome, the vitrectomy probe, through a limbal incision or with the phacoemulsification handpiece.[54]

The PFCLs are also helpful in retrieving intraocular lens by a similar method as for crystalline lenses. The intraocular lens will float on the PFCL bubble meniscus, **allowing for a secure grasp of the intraocular lens for repositioning** in the ciliary sulcus, removal through a limbal incision[48] or fixating to the sclera.[55,56]

Recently a modified technique for extracting a dislocated lens using perfluorocarbon liquids and viscoelastics has been described. This technique, consists of injecting an ophthalmic viscosurgical device around and on top of the heavy liquid bubble, and may contribute even further to the reduction of complications by keeping the lens centered and less mobile for easier phacofragmentation or removal by the cutting-suction probe.[57]

1.3.4 Suprachoroidal Hemorrhage

Suprachoroidal hemorrhages may occur intraoperatively or postoperatively. Experimental observations on a rabbit eye model suggests that maximum

liquefaction and the optimum time to drain both appear to be between 7 and 14 days due to increased retinal and ciliary body atrophy.[58] They also found that immediate sclerotomy during massive suprachoroidal hemorrhage is detrimental to the eye, with marked extension of the hemorrhage into the retina and vitreous.

Clinical data has shown that eyes with massive suprachoroidal hemorrhage can be treated successfully by secondary surgery.[59] Aggressive anterior and posterior segment reconstruction by anterior and posterior vitrectomy after sclerotomy drainage of the suprachoroidal hemorrhage is essential for better anatomic and visual results.[60]

PFCLs have been used on these approaches due to their posterior tamponade effect. This **pushes the liquefied suprachoroidal blood out** through the anterior sclerotomies.[60] Additional sclerotomies 4 mm posterior from the limbus can be created to significantly drain the suprachoroidal hemorrhage. PFCLs allow a more complete removal of suprachoroidal hemorrhage.[61]

1.3.5 Submacular Hemorrhage

Submacular hemorrhage may result from different etiologies, being the most common choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD).

Animal studies have demonstrated that irreversible photoreceptor damage can occur within 24 hours of submacular hemorrhage.[62] The visual outcome in eyes with submacular hemorrhages due to AMD is very poor as size and thickness of the hemorrhage negatively influence the natural prognosis.[63]

If left untreated, it can cause irreversible damage to the retinal pigment epithelium cells and retina.[64] Several surgical approaches involving the drainage or displacement of the hemorrhage have been described with improvement of vision.[65-74]

The recent trend has been toward the subretinal administration of recombinant tissue plasminogen activator (rTPA) after vitrectomy, followed by gas tamponade.[75] PFCLs may **facilitate removal of the liquefied submacular blood by displacing it away from the fovea**. Vitrectomy with peeling of the posterior hyaloid is followed by a posterior retinotomy near the edge of the hemorrhage and as far from the fovea as possible. Recombinant tissue plasminogen activator can then be injected on the subretinal space to try and liquefy the clot.[69,70,71] An anti vascular endothelial growth factor (VEGF) drug can be used in combination with rTPA in cases of submacular hemorrhage secondary to CNV.[72] A small bubble of PFCL is then injected over the macula to displace the remaining blood out of the macular area. The blood is then aspirated gently with a flexible-tipped cannula through the retinotomy site.

More recently autologous transplantation of the retinal pigment epithelium and choroid has been used to treat submacular hemorrhages secondary to CNV.[73,74] The PFCLs are used to **flatten the graft** before silicone oil exchange. Although promising, the surgical complications for this technique remain high.

1.3.6 Proliferative Diabetic Retinopathy

The PFCLs have been used in diabetic traction detachments either complicated or not by a iatrogenic retinal tear and rhegmatogenous diabetic retinal detachments.[76,77]

In diabetic traction retinal detachments, PFCLs can assist to: a) **separate membranes adherent to the retina**; b) **allow visualization and posterior diathermy of vessels during uncontrolled intraoperative bleeding**; c) **stop bleeding from the optic disc**; and d) **flatten the retina to allow a good endophotocoagulation**.[78,79] After vitrectomy, membranes should be addressed in a posterior-to-anterior direction. Following relief of all significant tractions a PFCL can be injected. This produces a better anatomic apposition of the retina to the retinal pigment epithelium and displaces the subretinal fluid in an anterior direction. A more peripheral retinotomy can then be done to drain the subretinal

fluid as persistent subfoveal fluid may take several months to resolve in patients undergoing pars plana vitrectomy to repair traction retinal detachment secondary to proliferative diabetic retinopathy and account for delayed visual recovery.[80] After the drainage of the subretinal fluid, panretinal laser endophotocoagulation is done. When used on these cases PFCLs **ensure retinal burns of uniform intensity**. When both traction and rhegmatogenous detachments are present, an encircling band may be placed to relieve traction.

Perfluorocarbon-perfused vitrectomy[30] and en bloc perfluorodissection[31] have been recently described and could help in cases of complicated tractional retinal detachments, but as previously described in section 1.3.1.2, prospective and comparative studies are necessary to establish formal indications and possible contra-indications of these techniques.

1.4 Clinical Outcomes

Most vitreoretinal surgeons acknowledge that the use of PFCLs has improved the management of complex retinal detachments and represent a major development in vitreoretinal surgery.[5,29] Although reliable scientific evidence of improved clinical outcomes is limited, the PFCLs have been adopted globally, as withholding the use of PFCLs in numerous cases would be inappropriate.[39]

Several large reports are available. In a study of 140 consecutive patients that underwent retinal detachment repair associated with proliferative vitreoretinopathy (PVR) grade C2 to D3 (American Retina Society Terminology Committee Classification) with perfluoroperhydrophenanthrene (Vitreon, Vitreophage, Lyons, Illinois), Carroll et al.[15] (The Vitreon Study Group) report a re-attachment rate at the final follow-up examination (mean 7 months) of 84%, and that 92% of patients had stable or improved visual acuity. The Vitreon Collaborative Study,[81] a multicenter prospective case series to evaluate the use of Vitreon on the management of giant retinal tears, enrolled 162 eyes of 161 patients with giant

retinal tears. At their most recent follow-up 147 (90.7%) eyes remained attached and visual acuity improved or remained stable in 105 (64.8%) eyes.

Coll et al.[16] report their study of 223 patients who underwent vitreoretinal surgery for severe PVR (93% D1-D3 - American Retina Society Terminology Committee Classification) where perfluoro-n-octane was used intra-operatively to flatten the retina, avoid posterior drainage retinotomy, to identify areas of residual retinal traction and retinal membranes, to stabilize the peripheral retina during dissection of anterior PVR, and to help determine the extent and location of relaxing retinotomies. After a single surgery 78% of the retinas were re-attached posterior to the scleral buckle and 96% were re-attached after multiple surgeries. An average of 1.24 vitrectomy surgeries were required. Final visual acuities were 20/400 or better in 74% of eyes and 20/80 or better in 30%.

The Perfluoron® Study Group reported two multi-centric studies of their outcomes with perfluoro-n-octane (Perfluoron, Alcon, Fort Worth, Texas) for PVR[82] and giant retinal tears.[83] These studies where used to obtain United States Food and Drug Administration (FDA) approval. The study for PVR[82] included 555 eyes of 555 patients followed up at a median of 5.6 months. At 6 months, the retina was attached in 279 (78%) eyes and visual acuity improved or remained stable in 380 (83%) eyes (percentages were based on the number of patients for whom the data was available at the time). The study for giant retinal tears[83] included 212 eyes of 212 patients followed a median of 3.5 months. At 6 months, the retina was attached in 108 (76%) eyes and visual acuity improved or remained stable in 151 (83%) eyes (percentages were based on the number of patients for whom the data was available at the time). Many smaller series reported better anatomic and visual outcomes for PVR and giant retinal detachments.[38,40] This variation might be due to case selection or inexperience with the surgical technique, as several of the surgeons on these two studies were inexperienced with the use PFCLs.[39]

The results of these large series compare favorably to other published studies where PFCLs weren't used.[84,85]

Currently there are a number of commercially available perfluorocarbon liquids for use in vitreoretinal surgery. Perfluoro-n-octane (Perfluoron®, Alcon, Fort Worth,

Texas, US) and Perfluorodecalin (DK-Line®, Bausch & Lomb, Rochester, New York) are the most common. The global market sales are divided between perfluoro-n-octane (55-60%) and perfluorodecalin (40-45%). Perfluoroperhydrophenanthrene (Vitreon®, Vitrophage, Lyons, Illinois, US) is also available but it is used by a very low percentage of surgeons (Mauro Beccaro, CEO at AL.CHI.MI.A, Padova, Italy, unpublished data). In the United States Perfluoron® is the perfluorocarbon liquid most commonly used.[86]

There are several reports comparing perfluoro-n-octane (PFnO) to perfluoroperhydrophenanthrene (PFPHP) and perfluorodecalin (PFD) for efficacy and complications.[87-89] In both published studies comparing PFnO to PFPHP[87,88] there was no statistically significant difference between the compounds on retinal re-attachment rates at 6 months. But there was a statistically significant higher rate of PFPHP retention observed post-operatively. This might be due to lower vapor pressure and PFCL interface visibility of the PFPHP. One study found that PFnO had a slightly better 6-month visual acuity and lower rates of corneal abnormality and elevated intraocular pressure.[87] Conversely, a second study concluded that there was a tendency at the 12-month follow-up examination for the cornea to remain clearer in the PFPHP group.[88]

Crafoord et al.[89] reported that no difference could be observed between PFnO and PFD, and that there were no adverse effects associated with their use. Furthermore Mathis et al. reported that PFD offered the advantage of low cost compared with PFnO.[90]

The theoretical advantage of PFnO compared to PFPHP is the higher vapor pressure and a refractive index greater than an aqueous or saline solution (Table 1, aqueous refractive index = 1.33). This allows for higher vaporization and interface visibility of the PFnO in relation to the PFPHP. PFD has intermediate characteristics between the PFnO and the PFPHP, while being less prone to form bubbles when injected into the eye, having a higher tamponading pressure and of lower commercial value. These are the characteristics that make PFnO and PFD the most used PFCLs worldwide.

1.5 Tolerance and Complications

1.5.1 Tamponade Use

1.5.1.1 Perfluorocarbon Liquid Used as Short Term Tamponade

Perhaps due to the idea that proliferative vitreoretinopathy (PVR) shows a predilection for the inferior fundus,[91] retinal surgeons have long been interested in a tamponade with a higher specific gravity than water for the inferior retina, that avoids the uncomfortable prone positioning that patients are subject to after surgery using a tamponade which is lighter than water.

Some anecdotal reports of PFCL short term use (≤ 14 days) for PVR[92] or giant retinal tears[93,94] seem to achieve good anatomic and functional results and suggest that PFCLs may be well tolerated for a short period. Although these approaches seem to be effective, they require an extra surgical procedure, as strategies reported involve making a silicone oil exchange on a considerable amount of patients.[92-94] Furthermore recurrence of retinal detachments caused by re-proliferation tended to occur on the superior quadrants when these strategies are employed.[95] Dispersion of PFCLs is also a concern as it has been reported to occur as early as 2-3 days after placement in the vitreous cavity of rabbits.[96,97] By preventing visualization of the retina through the bubbles, loss of optical clarity may result, and may allow small bubbles to pass through a retinal break. The rate of dispersion is related to factors such as turbulence, viscosity and solubility.[98]

1.5.1.2 Perfluorocarbon Liquid Used as Extended Term Tamponade

Leaving PFCLs in the vitreous cavity for short periods of time can be tolerated. However, when they are left for extended periods they can cause ocular toxicity and dispersion.[96] In addition, trabecular obstruction may be caused by macrophages that have ingested small PFCL bubbles. This could explain the reported complications such as inflammation and transient or persistent intraocular pressure elevation, related to the extended use of PFCLs as a tamponade.[99] Dispersion as described above is the formation of small bubbles of PFCL at the

PFCL-aqueous interface that can affect optical clarity or migrate to the subretinal space through retinal tears.

Most studies report that toxicity of PFCLs is related to either chemical toxicity or mechanical effects on the retina. Chemical toxicity is related to polar impurities present in PFCLs. The major impurities are hydrogen-based and result from the incomplete fluorination of hydrocarbons.[100] These charged hydrogen atoms may be responsible for alterations of the PFCL interface that permit the formation of a protein layer at the liquid-liquid interface.[101] Cells presumably do not react with the PFCL interface, but rather with the layer of protein formed at the interface, which ultimately promotes cell growth and membrane formation.[101-103] The purer the PFCL, the less capacity there is for cells to interact at the interface, as the presence of even small amounts of polar impurities can result in cell growth.[104] Consistent with the studies of Sparrow et al[104] are later reports comparing purified and non-purified PFD.[105,106] Furthermore not only might the degree of residual impurities unfavorably influence perfluorocarbon inertness, but the adsorption of lipoproteins and proteins by these active moieties may reduce interfacial tension such that the effectiveness of the tamponade and the threshold to emulsification is lowered.

Today commercially available PFCLs, namely perfluoro-n-octane, perfluorooctane and perfluorodecalin, are tested for residual impurities before commercialization. But there is still some confusion related to the difference between the United States FDA approved perfluoro-n-octane, which is 99% pure, and perfluorooctane, which is only 70-75% pure as it is a mixture of several PFCLs (Fig. 3), and for this reason cheaper than the FDA approved perfluoro-n-octane (Mauro Beccaro, CEO at ALCHIMIA, Padova, Italy, unpublished data).

The FDA approved PFnO has been studied in rabbits[97,107] and humans[93,94] with no damage occurring in the retina when left in the vitreous cavity for less than 7 days. When left in the eye for longer periods, inflammation developed and retinal changes were noted on histologic studies. Histologic changes reported are time dependent and appear to predominantly affect the outer retinal layers and Muller cells, and include photoreceptor drop-down (displacement of photoreceptor nuclei

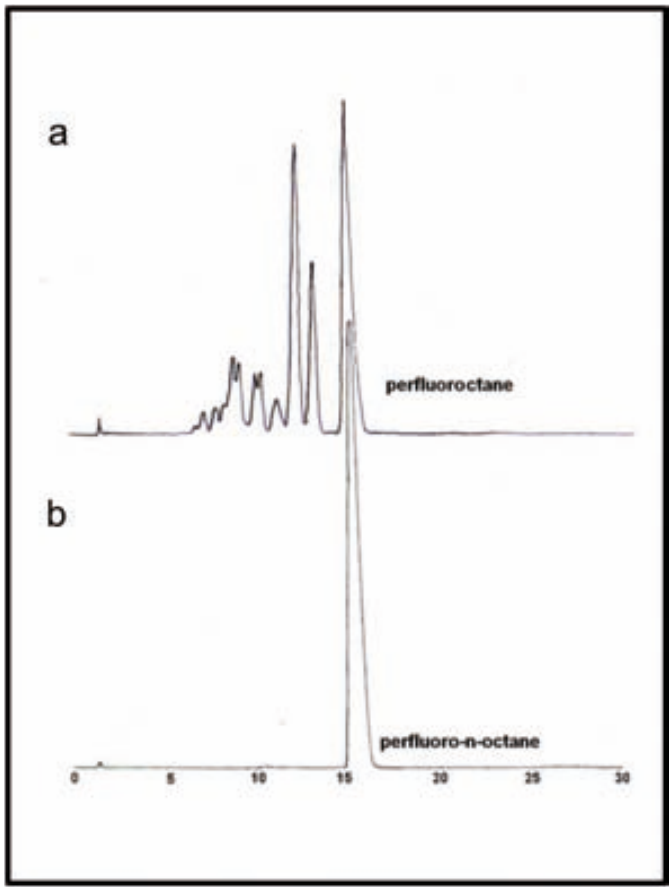

Fig. 3 Nuclear Magnetic Resonance (NMR) spectroscopic analysis of perfluorocarbon liquids (PFCLs) perfluorooctane and perfluoro-n-octane. With perfluorooctane (**a**) several spikes corresponding to the mixture with several other perfluorocarbons can be observed. When straight chain perfluoro-n-octane (**b**) is analyzed, a linear spike is observed thus confirming the purity of this PFCL.

into the rod and cone layer) commonly refered to as the moth-eaten phenomenon, narrowing of the outer plexiform layer, loss of photoreceptor inner and outer segments, hypertrophy and droplike protrusions of Muller cells and hypertrophy of retinal pigment epithelium.[97,107] These changes affect only the inferior retina. Similar changes in the superior retina have been described with intravitreal silicone oil in rabbit eyes,[108] and suggest a mechanical effect of the tamponading substance in the rabbit model.

PFD has also been studied in humans with no reported toxicity when left for less than 7 days,[92] but on rabbit eyes irreversible retinal damage involving the photoreceptors and retinal pigment epithelium was found after only 4 days of tamponade.[109] In long term tamponade in rabbits, PFD causes time dependent changes similar to the ones reported with PFnO that may progress to thinning of all layers of the retina by the fourth week and to localized areas of retinal atrophy in the inferior retina by the eighth week.[105,106,110] These more pronounced changes associated with PFD may be due to its higher specific gravity compared to PFnO. In regards to PFPHP there is some conflicting evidence as some studies have indicated a potential role of this PFCL as an extended-term replacement in the human eye,[46,111] but later studies contradict the previous studies by reporting retinal damage as a result of long-term use in both animals and humans.[112,113,114]

Another potential problem with using PFCLs as a post-operative intraocular tamponading agent involves the entrance of this compound in the anterior chamber, mainly on aphakic patients. Moreira et al.[115] reported stromal inflammation, corneal vascularization and "fish-egging" phenomenon (formation of small bubbles of PFCL on the anterior chamber) when 0.05 ml of PFCL were injected in the anterior chamber of rabbits for up to 14 days. Histology showed vacuoles of PFCL in the endothelium but not in the stroma, and loss of endothelial cells and retrocorneal membranes limited to the area of the cornea in contact with the PFCL. Based on these experiments they caution against short-term and long-term use of PFCLs as vitreous substitutes on aphakic patients.

1.5.1.3 Perfluorocarbon Liquid and Gas Tamponade

Meyer and Zak[116] examined the possibility of using a combination of PFPHP and perfluoropropane (C_3F_8) gas by determining the effect of the PFCL on the decay rate of intraocular C_3F_8 gas bubbles in the eyes of Dutch rabbits. The rate of decay of the gas bubbles was accelerated in eyes containing the PFCL, and so they concluded that C_3F_8 gas may be ineffective in eyes which contain PFPHP.

1.5.1.4 Perfluorocarbon Liquid and Silicone Oil Tamponade

Because silicone oil opposes the superior retina and PFCL the inferior retina, it supports both areas of pathology, being useful in the treatment of complex retinal detachments. The combined use of high and low specific gravity liquid vitreous substitutes has been studied in primates[117] and rabbits.[118] Sparrow et al. [118] concluded that a 2:1 ratio of silicone oil and PFCL would take advantage of the greater ability of the PFCLs to resist tractional retinal detachment while ensuring that the interface between the two liquids did not involve the visual axis. Because of its greater viscosity, silicone oil is less likely to enter the anterior chamber of aphakic eyes and may resist the movement of PFCL into the anterior chamber. Additionally, both studies suggest that silicone oil tends to delay the emulsification of PFCLs when both substances are used together as vitreous replacements.[117,118]

Conversely, in humans patients, Ciardella et al.[119] reported that PFCLs shouldn't be used with silicone oil as they tend to emulsify together, originating an opaque

fluid, which contains both silicone oil and dispersed PFCL bubbles. This could lead to inflammation and increase in intraocular pressure.

1.5.2 Intraoperative Use

Although PFCLs are well tolerated when used for vitreoretinal procedures, three main complications may occur with their use: 1. Retention in the vitreous cavity; 2. Migration to the subretinal space; 3. Migration to the anterior chamber.

1.5.2.1 Retention in the Vitreous Cavity

Small residual droplets of PFCL in the vitreous cavity have been described previously in this thesis. The retention rates of PFnO and PFD are similar and are normally bellow 8% in most series.[82,83,87,88,120,121] PFPHP has been reported to have retention rates as high as 30%.[87,122] Blinder et al.[122] recommended multiple partial air-fluid exchanges to reduce the retention rate and ensure a more complete removal of PFPHP. After this technique had been proposed some studies showed a reduced rate (8%) of retention with the PFPHP, although still statistically higher when compared to PFnO (4%).[88]

As explained in section 1.4, differences in the physical properties of PFnO and PFPHP may explain the significantly lower rate of intraocular retention with PFnO. First, the index of refraction of PFnO is 1.27, whereas the index of refraction of PFPHP is the same as that of saline and aqueous (1.33), making it difficult to distinguish from intraocular fluid intraoperatively. In contrast, the interface between PFnO and aqueous (or saline) is clearly identifiable because of the difference in refractive indices between the liquids. Second, the vapor pressures of PFnO and PFPHP are 52 mmHg and < 1 mmHg at 37°C, respectively. The higher vapor pressure of PFnO makes it more likely to evaporate and exit through the sclerotomy sites when in contact with air. Third, the viscosities of PFnO and PFPHP are 0.69 centistokes (cSt) and 8.03 cSt at 25°C, respectively. Because of the much lower viscosity, PFnO may be removed more readily than PFPHP.

A few residual droplets in the vitreous are usually well tolerated and have not shown to cause any deleterious effects.[6,38] However, young patients, patients with considerable residual vitreous gel, and eyes with larger amounts of retained PFCL may elicit a macrophage response, visible as a white flake-like material that deposits on the lens, ciliary body and peripheral retina.[123]

1.5.2.2 Migration to the Anterior Chamber

This normally occurs postoperatively when the bubbles retained in the vitreous cavity migrate to the anterior chamber, especially in aphakic patients. If PFCL residues migrate into the anterior chamber in an aphakic silicone oil filled eye, they may occlude the surgical coloboma of the iris at the 6-o'clock position, and produce a silicone-induced pupillary block glaucoma.[124]

It appears that short term corneal exposure to PFCLs can be tolerated. Green et al.[125] reported on injecting 50 microliters of PFnO in the rabbit anterior chamber; after seven days they found that the corneal endothelial permeability was unchanged and assumed that there was absence of toxicity for this substance should it reach the cornea either intra-operatively or post-operatively. But Moreira et al.,[115] reported on injecting the same quantity in the rabbit anterior chamber, but after 14 days found loss of endothelial cells and retrocorneal membranes limited to the area of the cornea in contact with the PFCL. Some years later Stolba et al.[126] published the same findings with PFD and PFPHP. Furthermore in humans there is evidence of inflammation of the cornea after prolonged contact with PFCLs, [127,128] producing irreversible changes that are only corrected by a penetrating keratoplasty. The changes normally include stromal edema with florid bullous keratopathy, inflammatory infiltration with vascularization, and deposition of PFCL within keratocytes and perivascular macrophages. Ramaesh et al.[129] have also described corneal epithelial toxic effects to subconjuntival PFCL. Based on these experiments it seems that corneal toxicity to PFCLs is time and contact-dependent. Therefore if small bubbles of PFCL are present in the anterior chamber, it is generally advisable to remove them using a 30G needle inserted through the limbus under topical anesthesia.

1.5.2.3 Migration to the Subretinal Space

This typically occurs intra-operatively when small bubbles of PFCL migrate through the peripheral tears, mainly in patients with complicated PVR when large peripheral retinotomies, especially if 360 degrees, are performed.[121] Normally reported subretinal migration rates of PFCL are relatively low (< than 4%),[87,120,130] but some series report rates as high as 11%.[121] Most of these reports regarding subretinal PFCL have been with 20-gauge (G) vitrectomy. Recently, there has been a trend towards small gauge transconjunctival microincision vitrectomy (23G or 25G) as many surgeons feel that these small gauge techniques decrease operating times, increase patient comfort, and reduce surgical trauma.[131,132,133,134] A recent small case report (12 eyes) of 23G and 25G vitrectomies for giant retinal tear detachments by Kunikata et al.,[135] revealed that the occurrence of subretinal PFCL migration could be increased with small gauge techniques by reporting a subretinal PFCL rate of 16.6% in their patients. One year later, Garg and Theventhiran[136] compared the retention rate of subretinal PFCL in 23G versus 20G vitrectomy for rhegmatogenous retinal detachment repair. They operated on 234 eyes, 176 being subjected to 20G surgery and 58 to 23G surgery, and found a 4.5-fold increase of subretinal PFCL in favor of 23G (10.6%) compared to 20G (2.3%) instruments. Furthermore an 8 times higher rate in the non-PVR 23G group (7.7%) compared with the non-PVR 20G group (0.9%) was also found. They attributed these findings to turbulent mixing of fluids, coupled with the low surface tension of the PFCL, generated many small bubbles, which might more easily pass through a standard peripheral break. Potential strategies to reduce the incidence of this complication included reducing the infusion pressure, clamping the infusion line, or using valved cannulas.

Although small amounts of subretinal PFCL outside of the macula are usually well tolerated and some reports point to the fact that some patients might recover visual acuity after its removal from under the fovea,[137,138] retained subretinal PFCL can be a serious complication as it causes toxicity to the retina and retinal pigment epithelium[139,140,141] and can result in a scotoma,[142] and permanent vision loss if present under the fovea.[143]

2. STUDY RATIONALE

2. STUDY RATIONALE

Perfluorocarbon liquids (PFCLs) where first developed as blood substitutes due to their inertness and extensive capacity for oxygen transportation.[1]

Since Chang et al.[5] described the use of PFCLs in humans in 1987 these compounds represent a major development in vitreoretinal surgery.[5,6,7,29,38] Their high specific gravity, low viscosity, immiscibility in water, and optical clarity make them particularly useful in the management of complex retinal detachments such as proliferative vitreoretinopathy (PVR)[6,9,15-17] and giant retinal tears[38,40,41] but they are also indicated for surgery secondary to rhegmatogenous retinal detachments without PVR,[10,11] ocular trauma,[7,42-45,47] dislocated intraocular and crystalline lenses,[48-53,57] suprachoroidal hemorrhage,[58,59,60,61] submacular hemorrhage,[75] proliferative diabetic retinopathy,[30,31,76-78,80] and also for a variety of intraocular surgical maneuvers like protecting retinal pigment epithelium from vital dyes and facilitating the removal of the internal limiting membrane in a retinal detachment secondary to a macular hole typically seen in high myopic patients.[144,145]

Most vitreoretinal surgeons acknowledge that PFCLs remain an invaluable tool that has greatly simplified the management of complex retinal detachments, especially giant retinal tears for which they are indispensable.[6,38]

Although reliable scientific evidence of improved clinical outcomes is limited, the PFCLs have been adopted globally. Several landmark reports are available, [15,16,81-83] some of them used to obtain United States Food and Drug Administration approval. Although the results of these large series compare favorably to other published studies where PFCLs weren't used,[84,85] many smaller series reported better anatomic and visual outcomes for PVR and giant retinal tear detachments.[38,40]

Although some reports suggest that PFCLs may be well tolerated for a short period (≤ 14 days), there are three issues raised by this approach. Firstly, they require an extra surgical procedure as many of the reported strategies involve making a silicone oil exchange on a considerable amount of patients.[92-94]

Secondly, the dispersion[96,97] of PFCLs is also a concern as it leads to loss of optical clarity, and may allow small bubbles to migrate to the subretinal space through a retinal break. Thirdly, the reported reproliferation occurring on the superior quadrants leading to superior retinal detachments.[95] If short term use of PFCLs might be tolerated, when left on the vitreous cavity for extended periods they can cause ocular toxicity, either from chemical or mechanical effects on the retina, dispersion, trabecular obstruction with transient or persistent ocular hypertension, inflammation and irreversible corneal decompensation with loss of endothelial cells.[96,99-110,112-115]

For the reasons previous described most surgeons use PFCLs almost exclusively as an intraoperative tool and removed it before concluding the surgery. But even if well tolerated when used only intraoperatively, there are potential complications with PFCL, mostly from extended unintended retention within the eye. Although retention rates as high as 30% have been reported for perfluoroperhydrophenanthrene,[87,122] the retention rates of perfluoro-n-octane and perfluorodecalin are similar and are normally bellow 8% in most series, [82,83,87,88,120,121] and this is one of the main reasons why these are the two most used PFCLs.

While a few residual droplets in the vitreous are usually well tolerated and have not shown to cause any deleterious effects,[6,38] larger amounts of retained PFCL, especially in young patients or patients with considerable residual vitreous gel, may elicit a macrophage response, visible as a white flake-like material that deposits on the lens, ciliary body and peripheral retina.[123] If these small PFCL bubbles migrate to the anterior chamber, they can produce time and contact-dependent corneal toxicity, that could lead to irreversible corneal decompensation. [115,141,142] And in an aphakic silicone oil filled eye, these small bubbles may even occlude the surgical coloboma of the iris at the 6-o'clock position, and produce a silicone-induced pupillary block glaucoma.[124]

However, a potentially more serious complication is when PFCL is retained in the subretinal space, due to the fact that subretinal PFCL seems to cause toxicity to the retina and retinal pigment epithelium[134-136] and can result in a scotoma,[137] and

in permanent vision loss if present under the fovea.[138] Intraoperatively, PFCL can be observed to migrate through large or stiff posterior retinal breaks, and its use is cautioned when there is substantial residual posterior traction and posterior break. Less well recognized, but evidently more common than the vitreoretinal surgeon realizes, is that PFCL can migrate progressively through peripheral breaks.

Typically reported subretinal migration rates of PFCL are relatively low (< than 4%),[87,120,125] but some series report rates as high as 11%.[121] One could think that these retention rates could have the tendency to improve as surgical techniques are more refined and surgeons become familiar with these compounds. The fact is that most of the published reports regarding subretinal PFCL have been done with 20-gauge (G) vitrectomy, and in the last few years, there has been a shift towards small gauge transconjunctival microincision vitrectomy (23G or 25G). When recently, Garg and Theventhiran[131] compared the retention rate of subretinal PFCL in 23G versus 20G vitrectomy for rhegmatogenous retinal detachment repair, they found a surprising 4.5-fold increase of subretinal PFCL when surgery was performed with 23G (10.6%) in comparison to 20G (2.3%) instruments. This means that instead of a decrease there may be an increase of subretinal PFCL migration due to the actual trend toward small gauge vitrectomy.

Most PFCL-induced complications occur mainly due to the transparent nature of these compounds. This particular characteristic makes it difficult to visualize and completely remove the PFCL during silicone oil and especially air exchanges. It also makes extremely difficult to visualize the PFCL on the subretinal space, particularly after silicone oil or air exchange, or in patients with suboptimal visualization. In light of these complications colored PFCLs could help to discriminate these interfaces, allowing direct visualization and a correct and safe removal of all the PFCL during air and silicone oil exchanges, including the subretinal space, minimizing its retention in the vitreous cavity.

3. HYPOTHESIS

3. HYPOTHESIS

The use of colored perfluorocarbon liquids (PFCLs) provides a better visualization of this compound during air and silicone oil exchanges and in the subretinal space. This allows for a more complete removal of PFCLs from the vitreous cavity during surgery, avoiding complications related with its intraocular retention and the potential migration to the subretinal space or anterior chamber, thus avoiding inflammatory and toxic effects when remaining inside the eye.

4. OBJECTIVES

4. OBJECTIVES

The objectives of the study were:

1. To evaluate the optimal stain concentration for each colored perfluorocarbon liquid (PFCL).

2. To evaluate the intra-operative behavior of colored PFCLs by comparing the different colored PFCLs with transparent perfluoro-n-octane under several surgical circumstances, including:

    a. Visualization under balanced salt solution in a retina stained with trypan blue.

    b. Visualization of instruments inside PFCLs.

    c. Visualization under air.

    d. Visualization under silicone oil.

    e. Visualization in the subretinal space.

    f. Determination of the influence of colored PFCLs on laser burns.

3. To observe the existence of silicone oil miscibility with colored PFCLs.

4. To evaluate the stability of colored PFCLs after endolaser photocoagulation.

5. To check whether colorant residue remained inside the eye following the use of colored PFCL.

6. To test the toxicity of the colorant used to stain the PFCL.

To achieve the proposed objectives, the study was divided into two phases. The first phase was intended to evaluate and select the preferred colored PFCL which had the best characteristics, regarding the first 5 objectives. It was performed mainly on enucleated pig eyes (“ex vivo”) and “in vitro”. The second phase utilized the selected colored PFCL to perform toxicity analysis on live pigs (“in vivo”). This methodology reduced the number of live animals that would have been necessary if toxicity analysis were performed for all colored PFCLs at our disposal. For

simplicity and continuity the division in phases will not be highlighted in the subsequent sections. Rather a sequential analysis of all objectives will be described.

5. MATERIAL & METHODS

5. MATERIAL AND METHODS

5.1 Colored and Transparent Perfluorocarbon Liquids

All colored and transparent perfluorocarbon liquids (PFCLs) used in this study were obtained from AL.CHI.MI.A (Padova, Italy) and could be sterilized by 0.22 μm polytetrafluoroethylene (PTFE) filtration. Two types of colored PFCLs were used (Fig. 4). The first type of PFCLs was directly colored by a blue phtalocyanine derivate dye [blue perfluoro-n-octane (PFnO) and blue perfluorodecalin (PFD)], while the remaining type was a mixture of a colored semi-fluorinated alkane (SFA) with other PFCLs (purple, red and yellow colored PFCLs). The latter were similar to the colored heavy liquids described by Rizzo et al.[146] The purple PFCL was excluded before the study began due to it's opacity.

Two types of dyes to color the PFCLs were at our disposal during the study. The red and yellow dye which is a polymerized benzindole derivate that is soluble in perfluoroalkyoxyoctane (red and yellow SFA + PFCL mixture) (Table 2), and a blue dye that is a fluorinated phtalocyanine derivate that is directly soluble in PFnO and PFD (directly colored blue PFnO and blue PFD), which physicochemical characteristics are similar to those described for transparent PFnO and PFD in Table 1, but with the addition of a blue coloring agent.

All the colored PFCLs were submitted to "in vitro" cytotoxicity tests according to the International Organization for Standardization (ISO) 10993-5:2009 on BalbC 3T3 cell culture. These studies were performed by AL.CHI.MI.A (Padova, Italy). On the basis of the results obtained, the colored PFCLs tested were considered not cytotoxic.

Table 2. Physicochemical Characteristics of some Colored PFCLs

Compound	Red PFCL	Yellow PFCL
Chemical formula	$C_{23}H_{15}F_{17}N{\bullet}C_4F_7O_2$	$C_{15}H_{15}N{\bullet}C_4HF_7O_2$
Molecular weight	841	423
Purity	> 95%	> 90%

Fig. 4. From left to right: yellow, red, purple and blue perfluorocarbon liquids (PFCLs). The yellow, red and purple dyes are a polymerized benzindole derivate and this dye is soluble in perfluoroalkyoxyoctane, a compound similar to the semifluorinated alkane perfluorohexyloctan, soluble with PFCLs and silicone oil and used to render it heavier-than-water. The blue dye is a fluorinated phtalocyanine derivate that is directly soluble in perfluoro-n-octane and perfluorodecalin.

5.2 Animals

5.2.1 Enucleated Pigs Eyes

Fresh enucleated pig eyes were obtained from a local slaughterhouse on the day of the wet lab experiments and stored in 4°C ice water to maintain corneal transparency.[147]

All enucleated pig eye experiments were performed at the wet lab of the Instituto de Microcirugía Ocular (IMO) in Barcelona (Spain), or at the wet lab of AL.CHI.MI.A in Padova (Italy).

5.2.2 Live Pigs

Hybrid pigs, weighing approximately 15 kg were used for this study. Pig eyes are frequently used in ophthalmologic studies,[148-151] due to the similarities between pig and human eyes in size and diameter, thickness of the retina, and laminar distribution of the capillary networks which supply the retina with blood.[152]

All live pig eyes experiments were performed in the operating room at Vall d'Hebron Research Institute (VHIR), where the animal facilities obey the legislation in force and are registered at the Departament de Medi Ambient i Habitatge with Registration Number B9900062. The live pig experiments performed obeyed the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research, and were submitted and approved by the Animal Research Ethics Committee of VHIR.

5.3 Procedures

5.3.1 Optimal Stain Concentration Test

This parameter was tested by an "ex vivo" experiment.

5.3.1.1 Study Design

After vitrectomy was performed on an enucleated pig eye, different concentrations of the available colored PFCLs were sequentially tested. This was repeated three times on three different enucleated pig eyes.

5.3.1.2 Study Setting

The surgical procedures were performed at the wet lab of the IMO in Barcelona (Spain).

5.3.1.3 Study Material

- An Accurus® Surgical System with an Accurus® Xenon Illuminator (Alcon/ Novartis AG, Basel, Switzerland) and a Möller-Wedel FS-1-12 surgical microscope (Haag-Streit, Koeniz, Switzerland) were used to perform vitrectomy.
- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.
- A Sony DXC-390P 3CCD Color Video Camera (Sony Corporation, Tokyo, Japan) connected to a JVC DV-Cam BR-DV6000 (JVC, Yokohama, Japan) were used to film the surgeries.
- Colored PFCLs were diluted in transparent PFnO and concentrations of 60%, 80% and 100% were obtained for every colored PFCL.
- Twelve enucleated pig eyes.

5.3.1.4 Study Method

Surgical Procedures

The eyes were observed with an indirect ophthalmoscope and excluded for media opacity and retinal detachment. The selected eye was then introduced into an artificial face model and placed under the microscope (Fig. 5). Then three 23-gauge (G) non-valved cannulas were inserted through the pars plana at a 30º oblique angle. One in the infero-temporal quadrant that connected to the infusion cannula and the other two placed in the supero-temporal and supero-nasal quadrants, at the 10 and 2 o'clock positions. All trocars were placed at approximately 3.5 mm of the limbus. At the beginning of the surgery triamcinolone acetonide was placed in the vitreous cavity to better visualize the vitreous. Under fiberoptic endo-illumination, the vitreous gel was removed as completely as possible and triamcinolone-assisted posterior vitreous detachment was performed. Upon completion of the vitrectomy the different concentrations of the desired colored PFCL were injected into the vitreous cavity.

The study of the different colored PFCLs was done in a sequential manner. The optimal concentrations of the colored PFCLs were tested in the following order: red PFCL, yellow PFCL, blue PFnO and blue PFD.

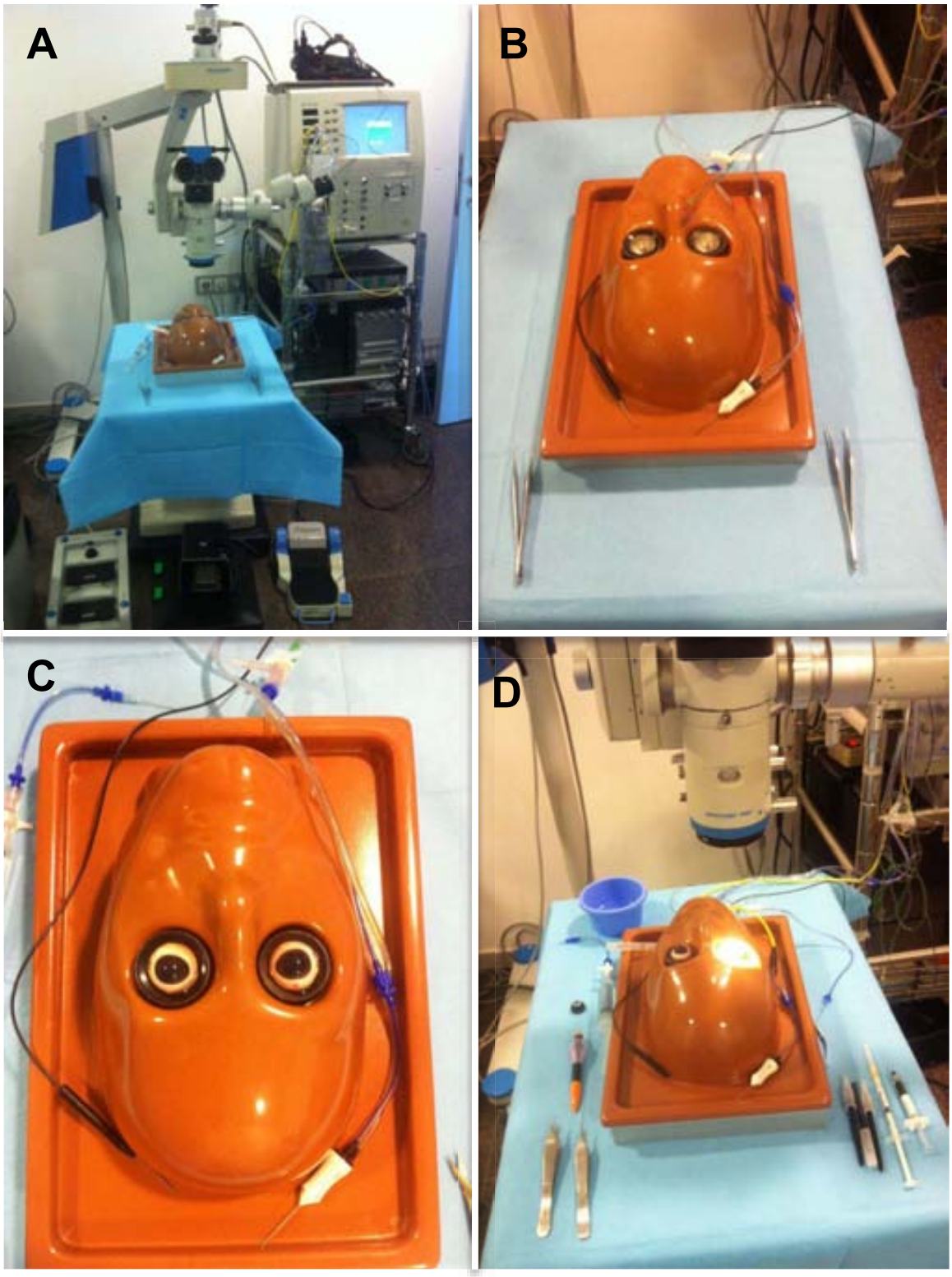

Fig. 5 An overall view of the material used to perform the “ex vivo” experiments **(A)**. A face model with a pig eye holder was used **(B)**. The pig eyes were then placed inside the holder **(C)** and the microscope then placed over the face model **(D)**.

5.3.2 Intraoperative Behavior Test

This parameter was tested by an “ex vivo” experiment, but the visualization in the subretinal space had to be complemented by an “in vivo” experiment. For the “in vivo” experiment approval by the Animal Research Ethics Committee of VHIR was obtained, as mentioned above on section 5.2.2.

5.3.2.1 Study Design

After vitrectomy was performed on an enucleated pig eye, several parameters were studied to evaluate the colored PFCLs intra-operative behavior. As these parameters were sequentially evaluated, the colored PFCLs that didn’t present the desired attributes were progressively excluded.

A protocol deviation occurred when studying the visualization of the PFCLs in the subretinal space. Due to the inability of studying this parameter on an enucleated pig eye, a live pig eye had to be used.

5.3.2.2 Study Setting

The surgical procedures were performed at the wet lab of the IMO and in the operating room at VHIR, in Barcelona (Spain).

5.3.2.3 Study Material

<u>At IMO wet lab and in the operating room at VHIR:</u>

- An Accurus® Surgical System with an Accurus® Xenon Illuminator (Alcon/ Novartis AG, Basel, Switzerland) and a Möller-Wedel FS-1-12 surgical microscope (Haag-Streit, Koeniz, Switzerland) were used to perform vitrectomy.
- A laser diode (Iridex, California, USA) was used to perform endolaser photocoagulation.
- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.

- A Sony DXC-390P 3CCD Color Video Camera (Sony Corporation, Tokyo, Japan) connected to a JVC DV-Cam BR-DV6000 (JVC, Yokohama, Japan) were used to film the surgeries.

- All four colored PFCLs, which best visible concentration had been previously determined, and the transparent PFnO were used.

- Twenty nine enucleated pig eyes were used to test this parameter at IMO wet lab, and two live pig eyes were used at the VHIR wet lab to complement the study of the visualization of the PFCL in the subretinal space.

5.3.2.4 Study Method

Surgical Procedures

The eyes were observed with an indirect ophthalmoscope and excluded for media opacity and retinal detachment. The selected eye was then introduced into an artificial face model and placed under the microscope (Fig. 5). Then three 23G non-valved cannulas were inserted through the pars plana at a 30º oblique angle. One in the infero-temporal quadrant that connected to the infusion cannula and the other two placed in the supero-temporal and supero-nasal quadrants, at the 10 and 2 o'clock positions. All trocars were placed at approximately 3.5 mm of the limbus. At the beginning of the surgery triamcinolone acetonide was placed in the vitreous cavity to better visualize the vitreous. Under fiberoptic endo-illumination, the vitreous gel was removed as completely as possible and triamcinolone-assisted posterior vitreous detachment was performed. Upon completion of the vitrectomy various maneuvers were performed according to the 6 parameters in study:

1. Visualization under BSS in a retina stained with trypan blue: An air-fluid exchange was performed and trypan blue dye was then injected over the posterior retina. After 2 minutes, the excess of dye was aspirated under air and a fluid-air exchange with removal of the residual dye was performed. Then 2 to 3 ml of the desired PFCL was introduced in the vitreous cavity. Afterwards the PFCL was removed and each tested sequentially.

2. Visualization under air: After the introduction of 2 to 3 ml of the desired PFCL in the vitreous cavity an air exchange was done first aspirating the fluid and after the PFCL. Then a fluid exchange was performed and the remaining tested sequentially.

3. Visualization of the instruments inside the studied PFCL: After the introduction of 2 to 3 ml of the studied PFCL in the vitreous cavity a 23G forceps was used to try to remove the internal limiting membrane. Afterwards the PFCL was removed and the others tested sequentially.

4. Visualization under silicone oil: After the introduction of 2 to 3 ml of the studied PFCL in the vitreous cavity a direct silicone oil exchange was done first aspirating the fluid and after the PFCL. To test a different PFCL another eye had to be used.

5a. Visualization in the subretinal space in the enucleated pig eye: Injection of 0.3 to 0.5 ml of the desired PFCL in the subretinal space was performed using a dual bore cannula. The other PFCLs were tested on a different location in the same eye, but only 3 PFCLs per eye could be tested. Subretinal visualization of the PFCLs wasn't possible to evaluate in an enucleated pig eye due to the opaque and thick nature of the dead retina, live pig eyes had to be used. The procedures in the live pig eyes are described in more detail in section 5b.

5b. Visualization in the subretinal space in the live pig eye: The study setting and material used to test this parameter in the live pig eye were the same as described in sections 5.3.6.2 and 5.3.6.3, respectively. But because this study was performed at a previous time and as a part of the intra-operative behavior testing, it is described here. The surgical method is the same as described in section 5.3.6.4 with the following changes: after vitrectomy with triamcinolone-assisted posterior hyaloid peeling, injection of 0.3 to 0.5 ml of the desired PFCL in the subretinal space was performed using a dual bore cannula. The other PFCLs were tested on a different location in the same eye, but only 3 PFCLs per eye could be tested. After the experiment the pigs were euthanized.

6. Determination of the influence of colored PFCLs on laser burns: After the introduction of 2 to 3 ml of the studied PFCL in the vitreous cavity, continuous and pulsed mode endolaser photocoagulation with a laser diode was performed. Afterwards the PFCL was removed and the others introduced sequentially with laser being done in a different location.

As described above, the study of the different colored PFCLs and the transparent PFnO was done in a sequential manner. First the colored PFCLs were used by the following order: red PFCL, yellow PFCL, blue PFnO and blue PFD; and after the transparent PFnO, except for points 4 (visualization under silicone oil) and 5 (visualization in the subretinal space). For point 4 a different enucleated pig eye was used for every time this experiment was performed. For point 5 live pig eyes had to be used due the inability to perform this test in enucleated pig eyes.

5.3.3 Silicone Oil Miscibility Test

This parameter was tested by an “in vitro” experiment at Standard Temperature and Pressure.

5.3.3.1 Study Design

Introduction of silicone oil in a vial with a colored PFCL. The vial was then closed and the mixture was observed after 10 minutes.

5.3.3.2 Study Setting

The laboratory procedures were performed at AL.CHI.MI.A wet lab in Padova (Italy).

5.3.3.3 Study Material

- An iPhone 4S camera (Apple, Cupertino, CA, USA) was used to photograph the experiment.
- Two vials with three colored PFCLs (yellow PFCL, blue PFnO and blue PFD), which best visible concentration had been previously determined.

- 1000 cSt silicone oil.

5.3.3.4 Study Method

"In Vitro" Procedure

- Approximately 3 ml of 1000 cSt silicone oil was introduced in a vial with 1 ml of colored PFCL.
- The vial was then closed and the mixture was observed and photographed after 10 minutes to allow the mixture to settle.

5.3.4 Stability Test

This parameter was tested by an "ex vivo" experiment.

5.3.4.1 Study Design

A vitrectomy with a laser stress test was performed on six enucleated pig eyes. Blue PFnO was used on three eyes and blue PFD on the other three eyes. After the test the colored PFCL was aspirated to a syringe and submitted to the stability spectrometric test.

5.3.4.2 Study Setting

The surgical procedures were performed on the wet lab of the IMO in Barcelona (Spain) and the laboratory procedures at AL.CHI.MI.A wet lab in Padova (Italy).

5.3.4.3 Study Material

At IMO wet lab:

- An Accurus® Surgical System with an Accurus® Xenon Illuminator (Alcon/ Novartis AG, Basel, Switzerland) and a Möller-Wedel FS-1-12 surgical microscope (Haag-Streit, Koeniz, Switzerland) were used to perform vitrectomy.

- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.
- A Sony DXC-390P 3CCD Color Video Camera (Sony Corporation, Tokyo, Japan) connected to a JVC DV-Cam BR-DV6000 (JVC, Yokohama, Japan) were used to film the surgeries.
- The two colored PFCLs (blue PFnO and blue PFD), which best visible concentration and best intraoperative behavior had been previously determined, and had no silicone oil miscibility issues, were used.
- Six enucleated pig eyes were used.

At AL.CHI.MI.A wet lab:

- Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometer was used to analyze the stability of the blue PFD and blue PFnO after the laser stress test.
- Nuclear Magnetic Resonance (NMR) spectroscopic analysis was used to confirm the results.
- Three syringes (2 ml capacity) filled with the blue PFnO and three with the blue PFD after the laser stress test, were analyzed.

5.3.4.4 Study Method

Surgical Procedures

The eyes were observed with an indirect ophthalmoscope and excluded for media opacity and retinal detachment. The selected eye was then introduced into an artificial face model and placed under the microscope (Fig. 5). Then three 23G non-valved cannulas were inserted through the pars plana at a 30º oblique angle. One in the infero-temporal quadrant that connected to the infusion cannula and the other two placed in the supero-temporal and supero-nasal quadrants, at the 10 and 2 o'clock positions. All trocars were placed at approximately 3.5 mm of the limbus. At the beginning of the surgery triamcinolone acetonide was placed in the

vitreous cavity to better visualize the vitreous. Under fiberoptic endo-illumination, the vitreous gel was removed as completely as possible and triamcinolone-assisted posterior vitreous detachment was performed. Upon completion of the vitrectomy, 2 - 3 ml of the desired PFCL was injected into the vitreous cavity and continuous endolaser photocoagulation performed during 15 min (power: 400 mW). After the laser stress test was done, the blue colored PFCL was aspirated to a 2 ml syringe. To repeat the test or test the other PFCL another eye had to be used.

The study of the 2 blue colored PFCLs was done in a sequential manner. First the test was performed 3 times in 3 different eyes with the blue PFnO, and then another 3 times with the blue PFD. The 6 syringes were then sent to AL.CHI.MI.A to be submitted to the stability spectrometric test.

Laboratory Procedures

- 60 µl of blue PFD (either laser-treated sample or reference material) was dried by nitrogen gas (N_2) flow at room temperature.
- After 30 minutes the solvent was completely removed and the residue was dissolved by adding 20 µl of PFnO.
- 2 µl of this solution was placed on the mass probe tip, gently dried by N_2 flow and MALDI-TOF EXPLORER 70107 analysis and then NMR spectroscopic analysis were performed.

5.3.5 Residue Test

This parameter was tested by an “in vitro” and an “ex vivo” experiment.

5.3.5.1 Study Design

The “in vitro” study consisted of preparing blue PFnO and blue PFD solutions and storing them at room temperature and at +4°C (refrigerator) for 14 days.

Microscopic evaluation was performed on solutions stored at room temperature and at +4°C (refrigerator) at Day 7 and Day 14.

The “ex vivo” study consisted on performing a vitrectomy with injection of the desired blue PFCL (blue PFnO or blue PFD) and an air-PFCL exchange at the end, on several enucleated pig eyes. After, the air-filled eyes were opened and microscopic evaluation of residue presence performed.

5.3.5.2 Study Setting

The “ex vivo” experiment was performed at the wet lab of the IMO in Barcelona (Spain) and repeated at the AL.CHI.MI.A wet lab in Padova (Italy), and the “in vitro” experiment only at the AL.CHI.MI.A wet lab.

5.3.5.3 Study Material

At IMO wet lab:

- An Accurus® Surgical System with an Accurus® Xenon Illuminator (Alcon/ Novartis AG, Basel, Switzerland) and a Möller-Wedel FS-1-12 surgical microscope (Haag-Streit, Koeniz, Switzerland) were used to perform vitrectomy.
- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.
- A Sony DXC-390P 3CCD Color Video Camera (Sony Corporation, Tokyo, Japan) connected to a JVC DV-Cam BR-DV6000 (JVC, Yokohama, Japan) were used to film the surgeries.
- Nine enucleated pig eyes were used for the “ex vivo” experiment.

At AL.CHI.MI.A wet lab:

- A Millennium™ Surgical System (Bausch & Lomb, Rochester, NY, USA) and a OPMI® pico/S100 surgical microscope (Zeiss, Oberkochen, Germany) were used to perform vitrectomy.

- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.
- A Leica DM IL LED Inverted Microscope with a Leica EC3 Camera connected to a Leica LAS Image Analysis software module (Leica, Wetzlar, Germany) were used to observe and photograph the existence of any dye residues, respectively.
- Four enucleated pig eyes were used to confirm the “ex vivo” experiment.
- Ten vials of blue PFnO and ten vials of blue PFD were used for the “in vitro” experiment.

In both wet labs:

- The two colored PFCLs (blue PFnO and blue PFD), which best visible concentration and best intra-operative behavior had been previously determined, and had no silicone oil miscibility issues, were used.
- The blue powder that remains after evaporation of the PFCL in contact with air was isolated and used.

5.3.5.4 Study Method

“In Vitro” Experiment

Preparation of Solutions

- The blue PFD solutions were prepared at an optimum concentration of 0.02% per volume of the blue dye using PFD as solvent. All solutions were prepared in the same manner, as follows:
  - ⋆ An adequate amount of powder raw material (blue dye) was weighted.
  - ⋆ The powder was solubilized with PFD solvent in a closed container; the solution was stirred by means of a magnetic stirrer. It took approximately three and a half hours to solubilize the dye completely.

  - ⋆ The solutions were checked, filtered with a 0.22 μm PTFE filter and sterilized in autoclave at 121°C for 30 minutes.
  - ⋆ The solutions were divided in clean glass vials, which were capped with chlorobutyl rubber stoppers and crimps.
- The blue PFnO solutions were prepared the same way.

Storage

- The solutions prepared as described above were stored at room temperature and at +4°C (refrigerator) for 14 days. A total five vials of blue PFnO and five vials of blue PFD were stored at room temperature, while the other five vials of blue PFnO and five vials of blue PFD were stored at +4°C (refrigerator).

Microscopic Evaluation

- Microscopic evaluation was performed on solutions stored at room temperature and at +4°C (refrigerator) at Day 7 and Day 14.
- Freshly prepared solutions served as control.

“Ex Vivo” Experiment

Design

The eyes were divided by three groups (three eyes per group):

1. Blue PFnO
2. Blue PFD
3. Blue Powder

Surgical Procedures

The eyes were observed with an indirect ophthalmoscope and excluded for media opacity and retinal detachment. The selected eye was then introduced into an artificial face model and placed under the microscope (Fig. 5). Then three 23G

non-valved cannulas were inserted through the pars plana at a 30º oblique angle. One in the infero-temporal quadrant that connected to the infusion cannula and the other two placed in the supero-temporal and supero-nasal quadrants, at the 10 and 2 o'clock positions. All trocars were placed at approximately 3.5 mm of the limbus. At the beginning of the surgery triamcinolone acetonide was placed in the vitreous cavity to better visualize the vitreous. Under fiberoptic endo-illumination, the vitreous gel was removed as completely as possible and triamcinolone-assisted posterior vitreous detachment was performed. Upon completion of the vitrectomy 2 - 3 ml of the desired colored PFCL or 0.05 ml of powder was injected into the vitreous cavity and then air exchange was performed, first aspirating the fluid and after the PFCL or powder. Then the scleral incisions were sutured and the air-filled eyes were fixed on 10% formaldehyde solution and sent to AL.CHI.MI.A (Padova, Italy) for microscopic residue testing.

Confirmation of the Experiment

Only 2 eyes per group to confirm the results of groups 1 and 2 were used at the AL.CHI.MI.A (Padova, Italy) wet lab. This was made due to doubts raised by the interference of the formaldehyde in PFCL evaporation.

The surgical procedures performed were exactly the same but the eyes were observed directly in the microscope without the need to use formaldehyde.

Microscopic Evaluation

- The globes were incised in the equator.
- The posterior lens capsule, peripheral and central retina of each globe were isolated.
- The presence of residues was evaluated by the stereomicroscope.
- Crystalline lens and retinal tissues were placed on Polysine Adhesion Slides Thermo Scientific 25x75x1 mm and analysed by the Leica inverted microscope.

5.3.6 Toxicity Test

This parameter was tested by an “in vivo” experiment.

5.3.6.1 Study Design

- The blue dye toxicity test was performed in several situations. The blue PFD was used in the study group and the transparent PFnO in the control group.
- Only the right pig eye was operated on as per protocol, but the left eye was also enucleated and studied histologically for comparative purposes.
- Three groups corresponding to different surgical procedures performed at Day 1 were established:
  - ⋆ Group 1 - Blue PFCL Toxicity: 1 to 1.5 ml of PFCL was left in a BSS filled eye, for one week.
  - ⋆ Group 2 - Blue Dye Residues Toxicity: 1 to 1.5 ml of PFCL was injected followed by an air exchange and a residual amount (0.05 to 0.1 ml) of PFCL was left in contact with air until vaporization and pigment residues left inside the eye, for one week.
  - ⋆ Group 3 - Blue PFCL Toxicity in the Subretinal Space: 0.05 to 0.1 ml of PFCL was left in the subretinal space of a BSS filled eye, for one week.
- Ophthalmologic examination was performed at Day 1 and every day after the surgical procedures and before euthanasia at Day 8.
- Electroretinography, color fundus photography and fluorescein angiography were performed on all eyes before the surgical procedures at Day 1 and before euthanasia at Day 8.
- Anatomopathological examination was performed on all eyes after euthanasia.

5.3.6.2 Study Setting

The surgical procedures were performed in the operating room at VHIR, in Barcelona (Spain).

5.3.6.3 Study Material

- An Accurus® Surgical System with an Accurus® Xenon Illuminator (Alcon/ Novartis AG, Basel, Switzerland) and a Möller-Wedel FS-1-12 surgical microscope (Haag-Streit, Koeniz, Switzerland) were used to perform vitrectomy.
- A Volk® MiniQuad XL surgical contact lens (Volk Optical, Ohio, USA) was used to visualize the posterior segment of the eye.
- A Sony DXC-390P 3CCD Color Video Camera (Sony Corporation, Tokyo, Japan) connected to a JVC DV-Cam BR-DV6000 (JVC, Yokohama, Japan) were used to film the surgeries.
- A TRC-50DX Retinal Camera (Topcon, Itabashi, Tokyo, Japan) was used to perform fundus photos and fluorescein angiographies.
- An HMsERG Model 2000 Full Field Flash Electroretinograph (OcuSciences, Ann Arbor, MI, USA) was used to perform electroretinography.
- A BX61 (Olympus, Shinjuku, Tokyo, Japan) fully automated microscope was used to perform histology.
- Eighteen eyes of eighteen hybrid pigs (≅15Kg) were used for the “in vivo” experiment.
- Only the blue PFD, which best visible concentration and best intraoperative behavior had been previously determined, and had no silicone oil miscibility or residue retention issues was used to perform the toxicity tests. Transparent PFnO was used in the control group.

5.3.6.4 Study Method

The procedures were performed the following way:

- At Day 1: The animals were anesthetized. Topical medication was then applied and ophthalmologic examination, electroretinogram, color fundus photography, fluorescein angiography and the surgical procedures were performed by the order described.
- From Day 1 to Day 8: Topical medication was applied and ophthalmological examination was performed.
- At Day 8: The animals were anesthetized. Topical medication was then applied and ophthalmologic examination, electroretinogram, color fundus photography, fluorescein angiography and euthanasia were performed by the order described.

All procedures performed are described bellow:

Anesthesia

The general anesthesia consisted of an induction with a mixture of tiletamine and zolazepan (3mg/kg), xylazine (2 mg/kg) and atropine (0.02 mg/kg) and maintained with the inhalation of isofluorene (1% to 2.5%) followed by endotracheal intubation. Mechanical ventilation was maintained with a fraction of inspired oxygen (FiO2) of 100%, with a maximum peak inspiratory pressure (PIPmax) of 20 cmH_2O, a tidal volume of 10 to 15 ml/kg, and an positive end-expiratory pressure (PEEP) of 5 cmH_2O. A neuromuscular blockade with cisatracurium (0.1 mg/kg) was done. The degree of anesthesia was monitored continuously with the assessment of the peripheral blood pressure, heart rate and rectal temperature.

Topical Medication

At Day 1 and Day 8, before all exams and surgical procedures the following eye drops were placed on all eyes:

- Nafazoline (0,5 mg/ml) + tetracaine (5 mg/ml) - for topical anesthesia.
- Phenylephrine hydrochloride (100 mg/ml) and tropicamide (10 mg/ml) - for pupil dilation.

- 5% povidone iodine solution - for disinfection.

At Day 1, after all surgical procedures the following eye drops were applied on all eyes:

- 5% povidone iodine solution - for disinfection.
- Chloramphenicol sodium succinate (7.3 mg/ml) + dexametasone disodium phosphate (1 mg/ml) - as antibiotic and anti-inflammatory.

Between Day 1 and 8, the following eye drops were placed on all eyes:

- Tropicamide (10 mg/ml) - for pupil dilation.
- Chloramphenicol sodium succinate (7.3 mg/ml) + dexametasone disodium phosphate (1 mg/ml) - as antibiotic and anti-inflammatory.

Ophthalmologic Examination

The ophthalmologic examination consisted of performing inspection of the eye and surrounding tissues and indirect binocular ophthalmoscopy. It was recorded the status of the cornea, lens, vitreous cavity and retina. Examination was performed daily.

Electroretinograms

After 30 minutes of dark adaptation, full field electroretinograms were recorded (Fig. 6). The HMsERG Model 2000 Full Field Flash Electroretinograph (OcuSciences, Ann Arbor, MI, USA) automatic protocol in accordance with the International Society for Clinical Electrophysiology of Vision (ISCEV) was performed. It controls the flash intensity, flash frequency, number of flashes to be averaged, the background illumination, and, if required, the dark and/or light adaptation time for each step. A

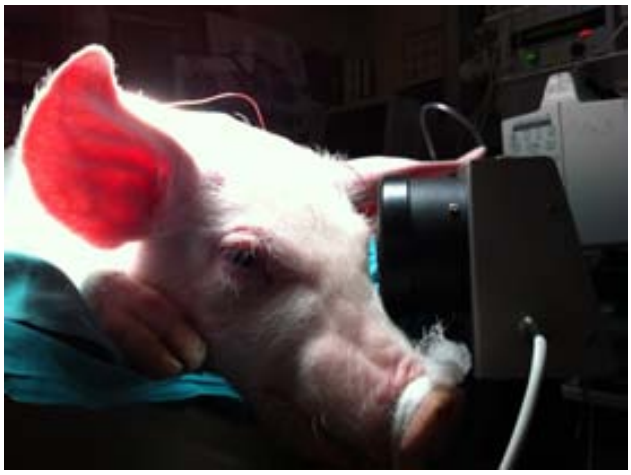

Fig. 6 Pig positioning for electroretinogram.

Ganzfeld type 76 mm diameter flash dome with 55 mm aperture was used as stimulator. This provides uniform illumination of the eye as required by ISCEV-2008 with intensity from 10 mcd.s/m^2 to more than 30 cd.s/m^2.

Color Fundus Photography

Color fundus photography was obtained on both eyes before performing fluorescein angiography. To perform the color fundus photographs and fluorescein angiography another investigator was needed to position the head.

Fluorescein Angiography

To perform angiography 2ml of a 10% fluorescein sodium solution were administered intravenously in the marginal ear vein. The photographs were made before the display of fluorescein in the choroidal vessels and was continued at intervals of 2 seconds for the first 40 seconds. After the photograph frequency decreased and the late phase was obtained after 5 minutes.

Surgical Procedures

The animals were placed in the supine position and the head positioned and fixed so that the cornea was horizontal. The skin hairs surrounding the eye and eyelashes were cut. The surrounding skin area was disinfected with 10% povidone iodine solution. An operating field with a hole was placed and a eyelid speculum was used to maintain the lids opened. When necessary a lateral canthotomy to facilitate exposure of the sclera was performed and sutured at the end of the surgery.

Then three 23G non-valved cannulas were inserted through the pars plana at a 30º oblique angle. One in the infero-lateral (infero-temporal) quadrant that connected to the infusion cannula and the two other were placed in the dorso-lateral (supero-temporal) and dorso-medial (supero-nasal) quadrants, at the 10 and 2 o'clock positions. All trocars were placed at approximately 2 mm of the limbus. At the beginning of the surgery triamcinolone acetonide was placed in the vitreous cavity to better visualize the vitreous. Under fiberoptic endo-illumination,

the vitreous gel was removed as completely as possible and triamcinolone-assisted posterior vitreous detachment was performed.

Upon completion of the vitrectomy various maneuvers were performed according to the 3 parameters in study:

1. Blue PFCL Toxicity: Injection of 1 to 1.5 ml of the desired PFCL was performed and then the scleral incisions were sutured with a transconjuntival 7-0 vycril suture.

2. Blue Dye Residues Toxicity: After the introduction of 1 to 1.5 ml of the desired PFCL in the vitreous cavity an air exchange was done first aspirating the fluid and after the PFCL. A residual amount (0.05 to 0.1 ml) of PFCL was left in contact with air until vaporization and pigment residues were left inside the eye. Then the scleral incisions were sutured with a transconjuntival 7-0 vycril suture.

3. Blue PFCL Toxicity in the Subretinal Space: Injection of 0.05 to 0.1 ml of the desired PFCL in the subretinal space inferior to the optic disc was performed using a dual bore cannula. Ringer lactate was left inside the eye. Then the scleral incisions were sutured with a transconjuntival 7-0 vycril suture.

In each group, six eyes were used (three eyes with blue PFD and three eyes with transparent PFnO). All the animals (eyes) were followed for 7 days and then were euthanized.

Euthanasia

The animals were euthanized with an overdose of intravenous pentobarbital sodium at the end of Day 8. After euthanasia the eyes were enucleated and fixed in 10% formaldehyde for minimum 24h at room temperature.

Light Microscopy

The fixed eyes were coated with paraffin after being fixed in 10% formaldehyde for minimum 24h at room temperature. Then 3 μm sequential cuts were made and 10 sections per eye were obtained and stained with hematoxylin-eosin to evaluate:

- **Retinal integrity and morphological abnormalities:** Morphometric and histological analysis were performed on all 10 sections obtained per eye.

- **Retinal ganglion cell number:** Ganglion cells were counted in 5 fields (40X magnification) of the central and peripheral inferior retina in the 10 sections per eye.

- **Correlation of the thickness of several retinal layers:** The thickness measures of the retinal layers were made using the ImageJ software. Measurements from three distinct areas (20X magnification) of the central and peripheral inferior retina were obtained.

- **Apoptosis:** Performed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which is a method for detecting DNA fragmentation that results from apoptotic signaling cascades.

6. RESULTS

6. RESULTS

A total of 60 enucleated pig eyes and 20 live hybrid pig eyes were used to test the the colored perfluorocarbon liquids (PFCLs) best visible concentration, intraoperative surgical behavior, silicone oil miscibility, stability, dye residue retention and toxicity.

The results obtained for each one of these experiments are described bellow.

6.1 Optimal Stain Concentration Test

As described on section 5.3.1.4 an “ex vivo” experiment with enucleated pig eyes was performed to evaluate the best visible concentration for colored PFCLs. Different concentrations were tested after the colored PFCL was filtered by a 0.22 μm polytetrafluoroethylene (PTFE) filter. The optimal concentration was defined as the concentration for which the PFCL was clearly distinguishable from balanced salt solution (BSS), air or silicone oil while maintaing adequate transparency, which provided adequate differentiation to allow work on the retinal surface. For the red colored PFCL the best visible concentration was an 80% dilution and for blue and yellow colored PFCLs no dilution was necessary, so the 100% filtered solution was used. These colored PFCL concentrations were used during all remaining experiments.

6.2 Intra-operative Behavior Test

After vitrectomy was performed on an enucleated pig eye, several parameters were studied to evaluate the colored PFCLs intra-operative behavior, as described on section 5.3.2.4. As these parameters were sequentially evaluated, the colored PFCLs that didn’t meet the desired attributes were progressively excluded.

6.2.1 Visualization Under BSS in Retina Stained with Trypan Blue

All colored PFCLs interfaces with BSS were clearly more visible than that of the transparent perfluoro-n-octane (PFnO). With the exception of red PFCL, the stained retina, optic nerve and blood vessels were clearly seen with all the other colored PFCLs, although the colored PCFLs did introduce some clarity degradation compared to transparent PFnO (Fig. 7).

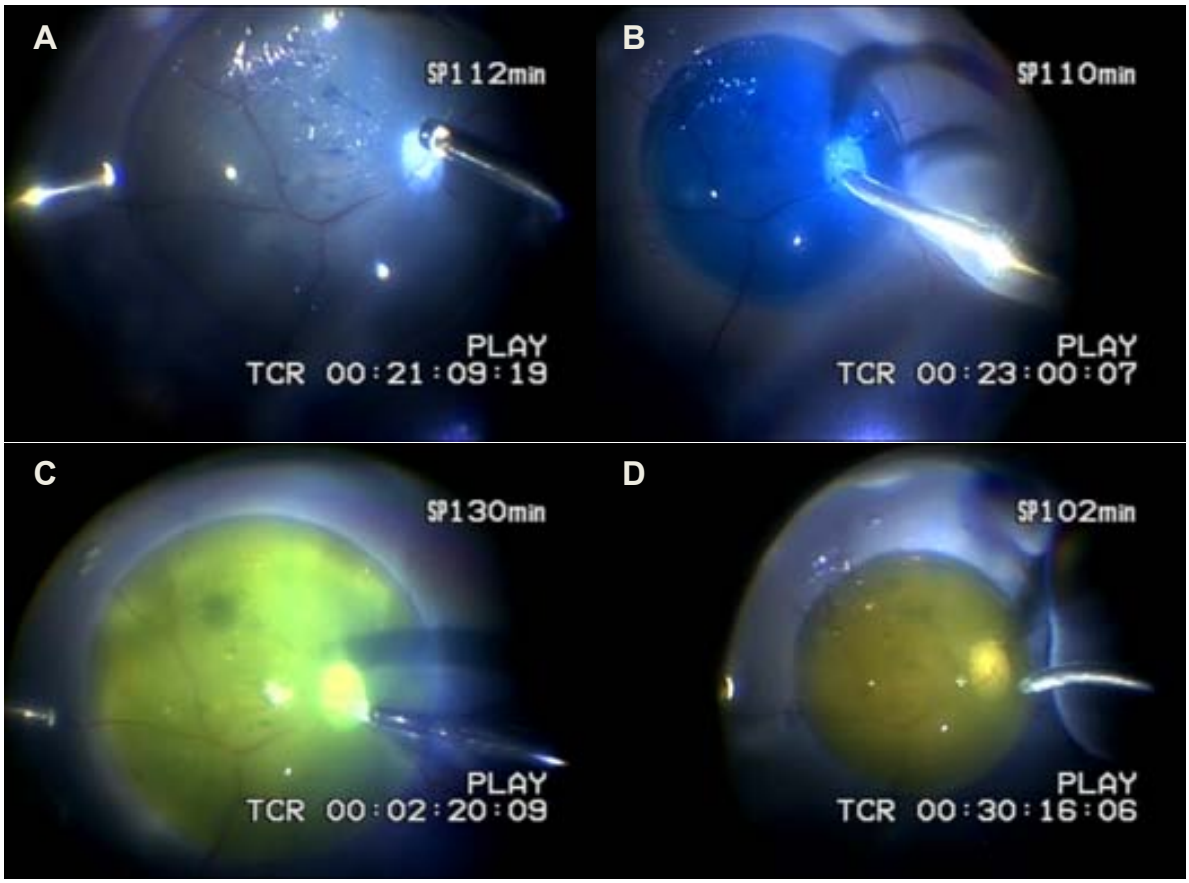

Fig. 7 Interfaces of **(A)** transparent perfluoro-n-octane (PFnO), **(B)** blue perfluorodecalin, **(C)** yellow and **(D)** red perfluorocarbon liquids (PFCLs) with a balanced salt solution. The underlying retina is stained with trypan blue and although the retina, optic disc and vessels can be distinguished with the red PFCL **(D)**, the image is blurred. The image of the same structures with blue **(B)** and yellow **(C)** PFCLs are clear even though not as clear as with the transparent PFnO **(A)**.

6.2.2 Visualization Under Air

All colored PFCLs were better visualized under air than the transparent PFnO (Fig. 8). Although the interface with air of the transparent PFnO could be estimated by

the reflex produced by the soft tipped cannula when entering the PFCL, it could not be seen. When the reflex ended due to the diminished level of PFCL inside the eye, it was difficult to know which residual quantity of transparent PFCL remained inside the eye. Further aspiration around and adjacent to the disc was performed with the soft tipped cannula in contact with the optic disc and retina and more PFCL could be aspirated. It was assumed that the residual PFCL that remained inside the eye would be vaporized in contact with air.

With the colored PFCLs the interface with air could be clearly seen and apparently all PFCL could be aspirated, even when residual quantities of these colored PFCLs remained inside the eye (Fig. 8).

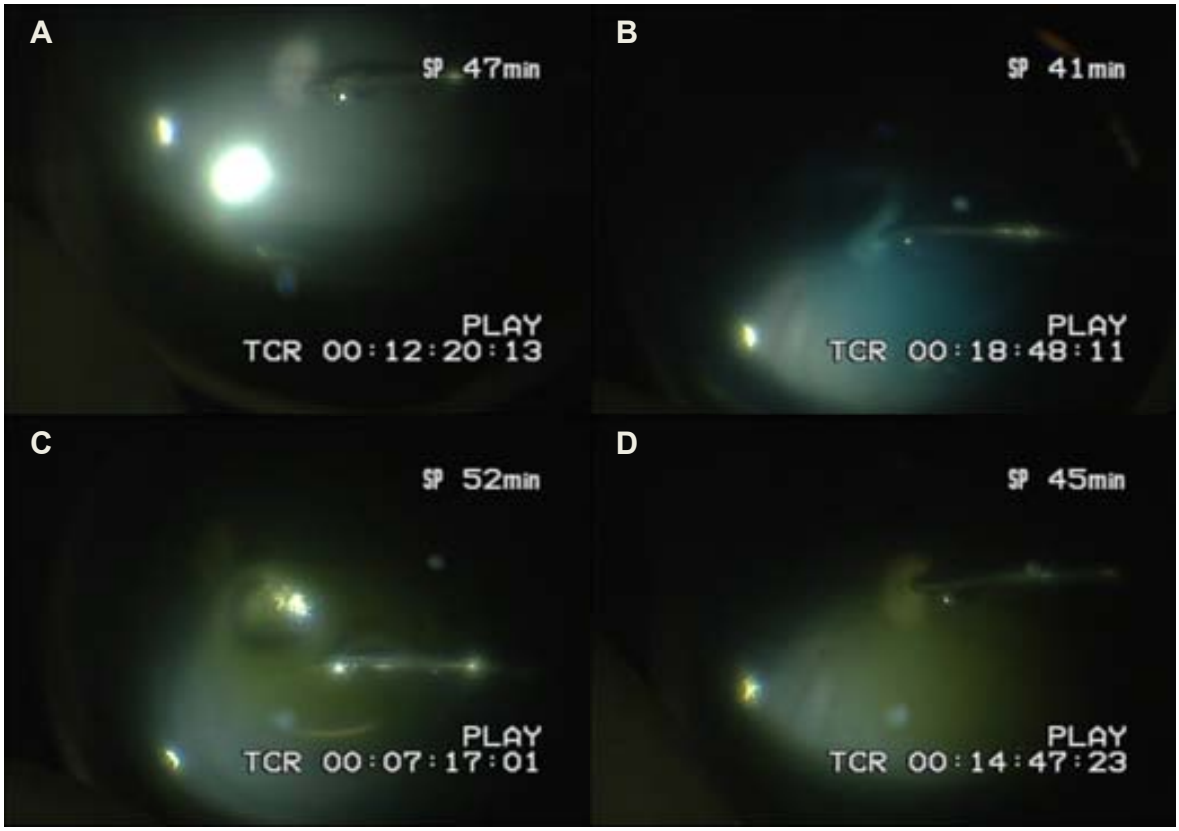

Fig. 8 Interfaces of **(A)** transparent perfluoro-n-octane (PFnO), **(B)** blue perfluorodecalin, **(C)** yellow and **(D)** red perfluorocarbon liquids (PFCLs) with a air. The interfaces with air of all colored PFCLs [**(B)**, **(C)** and **(D)**] are clearly better distinguished than the one with the transparent PFnO **(A)**, which is only estimated by the reflex produced by the soft tipped cannula entering the PFCL.

Because red PFCL wasn't superior to the blue and yellow colored PFCLs it was excluded at this stage, and no further testing with the red PFCL took place.

6.2.3 Visualization of the Instruments Inside the Studied PFCL

The blue perfluorodecalin (PFD) and yellow PFCL permitted a visualization similar to that obtained with the transparent PFnO (Fig. 9), and allowed manipulation of retinal surface without apparent restrictions. Although the retina was stained with a blue dye (trypan blue) and the PFD was also blue colored, that didn't appear to affect the ability to see the stained retina, once the shades of blue were different. Blue PFnO showed the same results as blue PFD.

Fig. 9 Visualization of the instruments and ability to work on the retinal surface. **(A)** Transparent perfluoro-n-octane, **(B)** blue perfluorodecalin and **(C)** yellow perfluorocarbon liquid (PFCL). Although not as clear as transparent PFnO **(A)**, blue **(B)** and yellow **(C)** PFCLs allowed a correct visualization of the instruments and manipulation of the retinal surface.

6.2.4 Visualization Under Silicone Oil

The interface of the colored PFCLs (blue and yellow PFCLs) with silicone oil was better distinguished from that formed between the silicone oil and transparent PFnO (Fig. 10). There was no change on the blue PFD and transparent PFnO interfaces with time, but the yellow PFCL exhibited a loss of perception of the interface (after approximately 3 to 5 minutes) and then a diffusion of the dye into the silicone oil (after approximately 5 minutes). If the yellow PFCL wasn't aspirated, with time a greater portion of the silicone oil in contact with the PFCL would become colored. This finding lead us to perform an "in vitro" experiment which material and methods were described on section 5.3.3 and the results are described separately on section 6.3. Based on these and the "in vitro" experiment

results, the yellow PFCL was excluded at this stage, and no further testing with the yellow PFCL took place. Blue PFnO showed the same results as blue PFD.

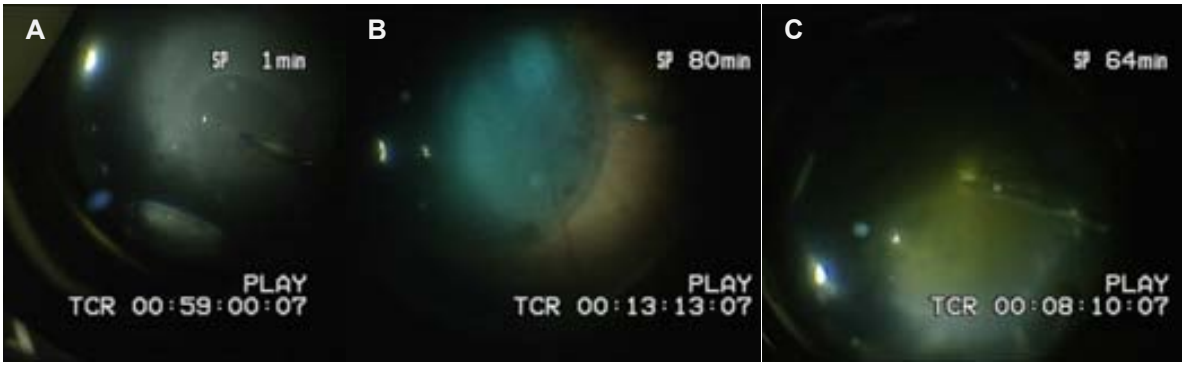

Fig. 10 Visualization of a direct silicone oil - perfluorocarbon liquid (PFCL) exchange. **(A)** Transparent perfluoro-n-octane (PFnO), **(B)** blue perfluorodecalin and **(C)** yellow PFCL. Blue PFD interface **(B)** is better visualized than transparent PFnO **(A)**. With the yellow PFCL **(C)** we can observe a temporal relationship as the interface loses definition and diffusion of the dye into the silicone oil occurs (after approximately 5 minutes).

6.2.5 Visualization in the Subretinal Space

As described on section 5.3.2.4, due to the opaque and thick nature of the enucleated pig eye retina, that occurs a few hours after it's enucleation, the subretinal visualization of the PFCLs was no longer possible to evaluate (Fig. 11). Due to this effect, a separate experiment involving live hybrid pig eyes, also described in section 5.3.2.4, was performed, requiring additional approval from the Animal Research Ethics Committee at VHIR.

After the injection of colored and transparent PFCLs into the subretinal space of live hybrid pigs, only the blue PFD and blue PFnO were clearly visualized on the subretinal space (Fig. 12). Transparent PFnO could not be visualized on the subretinal space. As three PFCLs could be injected per eye, the yellow PFCL was also used on this particular experiment, but the results were similar to those obtained with the transparent PFnO (Fig. 12).

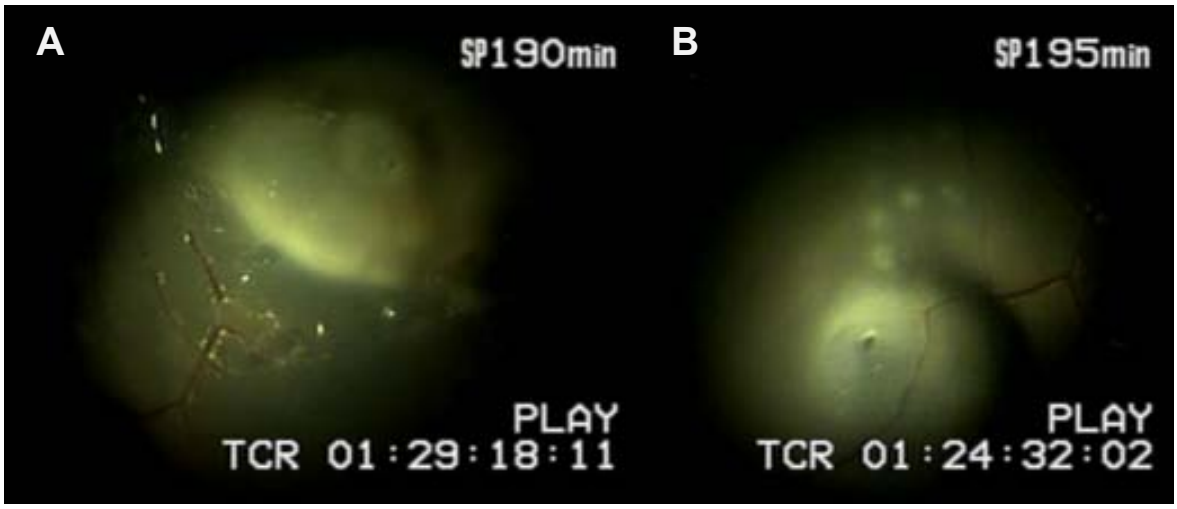

Fig. 11 Visualization of **(A)** transparent perfluoro-n-octane and **(B)** blue perfluorodecalin in the subretinal space of an enucleated pig eye. On both images it is clear the opaque and thick nature of the enucleated retina that prevents the correct visualization of the PFCLs. This is likely due to postmortem stasis.

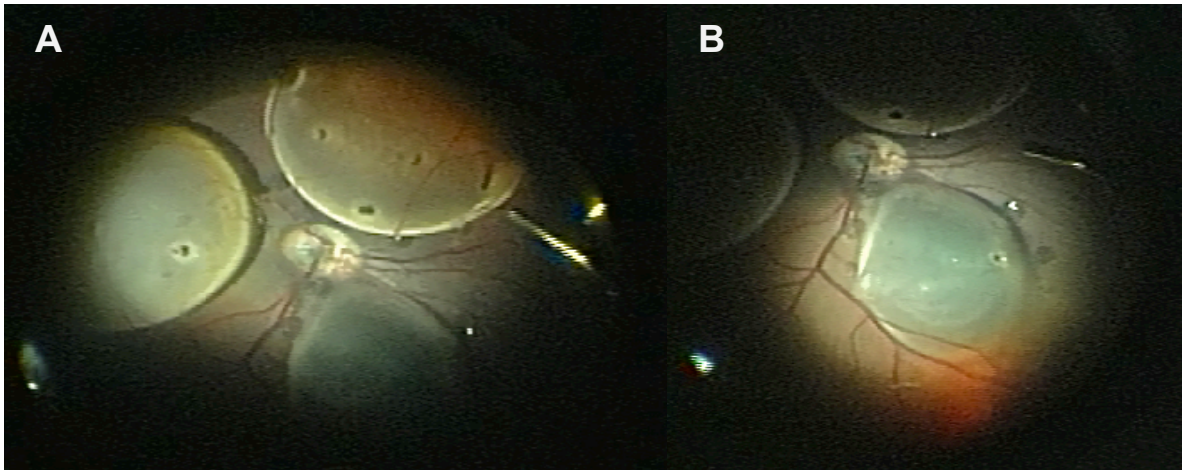

Fig. 12 Visualization of transparent and colored perfluorocarbon liquids (PFCLs) in the subretinal space of a live hybrid pig eye. **(A)** Transparent perfluoro-n-octane can be seen on the left, yellow PFCL on the top and blue perfluorodecalin (PFD) on the bottom. On **(B)** blue PFD can be better observed. When comparing both images it becomes clear that the blue PFD is the only PFCL that can be clearly visualized in the subretinal space.

6.2.6 Determination of Influence of colored PFCLs on Laser Burns

Continuous and pulsed modes were performed to evaluate this parameter. In continuous mode, although more time to produce a laser burn was needed for blue PFD, it didn't seem to significantly affect the intensity or visualization of the laser burn (Fig. 13). On pulsed mode it was necessary to increase the duration time by

300 to 400 ms to obtain the same burn effect as when performing laser through BSS. It was also observed that increases of 200 to 300 mW of power reduced time consumption if extensive laser was necessary.

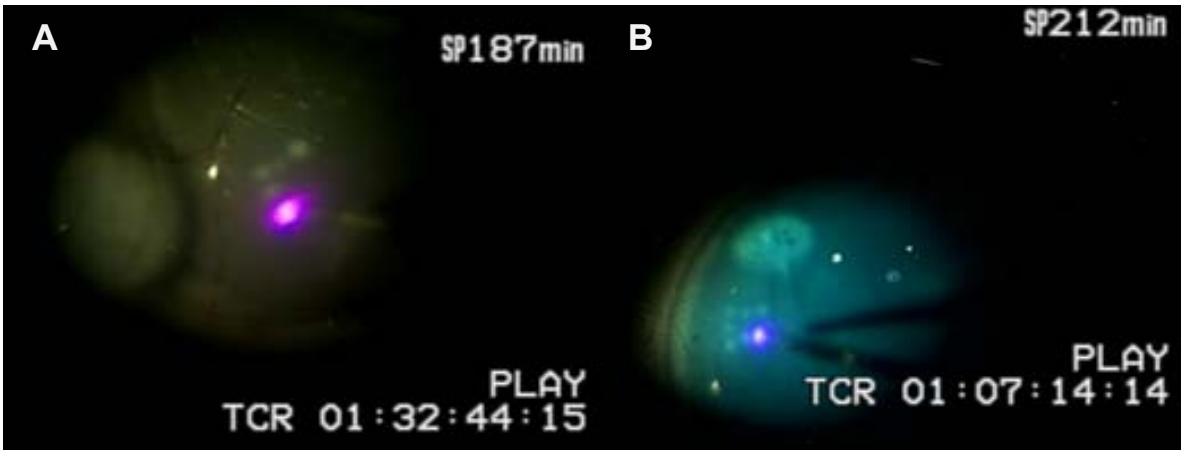

Fig. 13 Visualization of laser burns with **(A)** transparent perfluoro-n-octane (PFnO) and **(B)** blue perfluorodecalin (PFD). On both images [**(A)** and **(B)**] the laser burns can be clearly identified. Although less amount of energy was necessary to produce a laser burn with transparent PFnO **(A)**, laser performance through the blue PFD **(B)** was not a problem.

A summary of the study results of the intra-operative behavior test for all colored PFCLs can be seen in Table 3.

Table 3 - Intra-Operative Behavior Results

	Red PFCL	Yellow PFCL	Blue PFD	Blue PFnO	Transp.* PFnO
Under BSS	+	+++	+++	+++	++
Under Air	+++	+++	+++	+++	-
Instruments	excluded	++	++	++	+++
Under SiO	excluded	-	+++	+++	++
Subretinal	excluded	-	+++	+++	-
Laser	excluded	excluded	++	++	+++

* Transparent

6.3 Silicone Oil Miscibility Test

This “in vitro” experiment was performed due to the perception of diffusion of the yellow PFCL dye into the silicone oil during a direct exchange, as described on section 6.2.4. After approximately 8 minutes from the introduction of the silicone oil on the yellow PFCL vial, the vial content acquired the same color (Fig. 14). Only one phase could be observed, which inferred that yellow PFCL is miscible with silicone oil. In the blue PFD vial no miscibility between the PFCL and the silicone oil was detected. The two separate phases could be observed even after 10 minutes (Fig. 14).

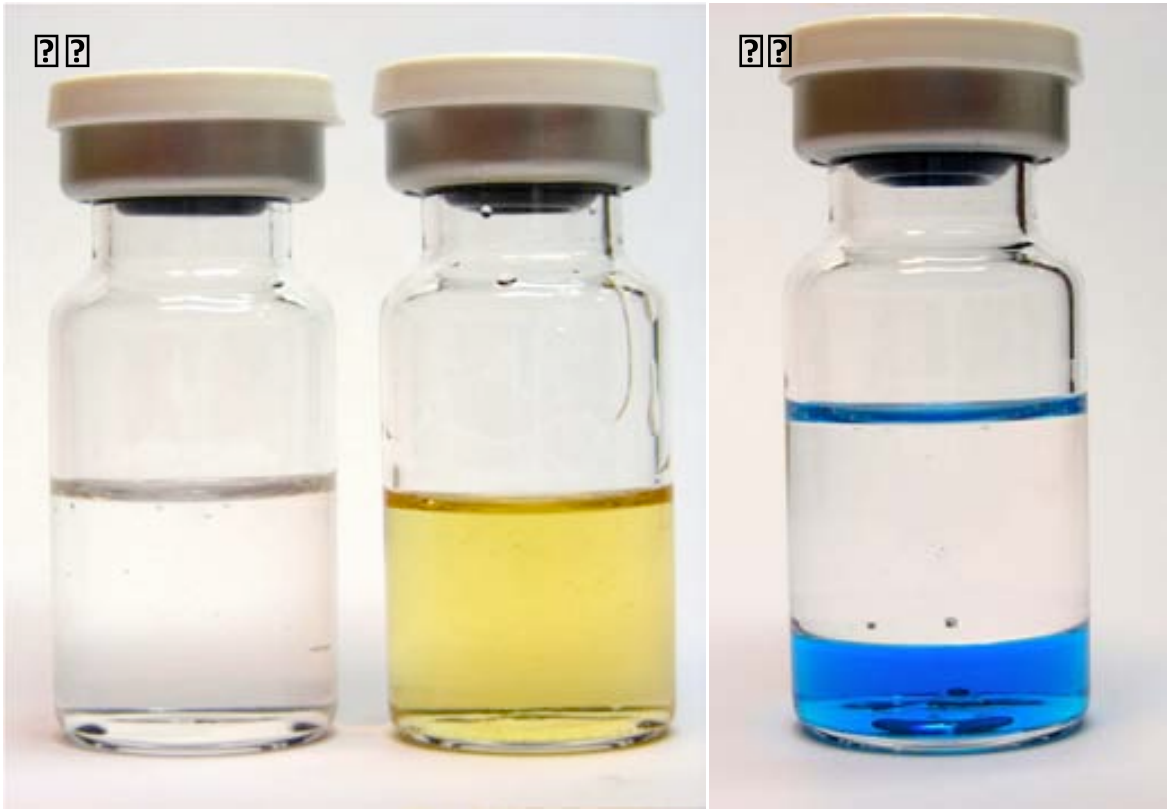

Fig. 14 Silicone oil miscibility test of colored perfluorocarbon liquids (PFCLs). **(A)** A vial with 1000 cSt silicone oil can be seen on the left. On the right a vial with a yellow compound (only one phase observed) that resulted from the introduction of the silicone oil in the vial with yellow PFCL. This photo was taken after 10 minutes of the introduction of the silicone oil, demonstrating the miscibility of the yellow PFCL with silicone oil. **(B)** A vial with blue perfluorodecalin (PFD) after 10 minutes of the introduction of silicone oil. In the vial 2 distinct phases can be observed, demonstrating the immiscibility of the blue PFD in silicone oil at Standard Temperature and Pressure.

6.4 Stability Test

After vitrectomy was performed on an enucleated pig eye, blue PFD and blue PFnO were submitted to a laser stress test, aspirated into a syringe and then analyzed to see if the physicochemical characteristics remained the same, as described on section 5.3.4.

MALDI-TOF EXPLORER 70107 analysis did not show alteration of both blue colored PFCLs analyzed when comparing them before and after the laser stress test. This was confirmed by Nuclear Magnetic Resonance (NMR) spectroscopic analysis that showed the same results (Fig. 15). As both analysis were consistent and produced the same results on all analyzed samples, stability of the blue PFD and blue PFnO was confirmed.

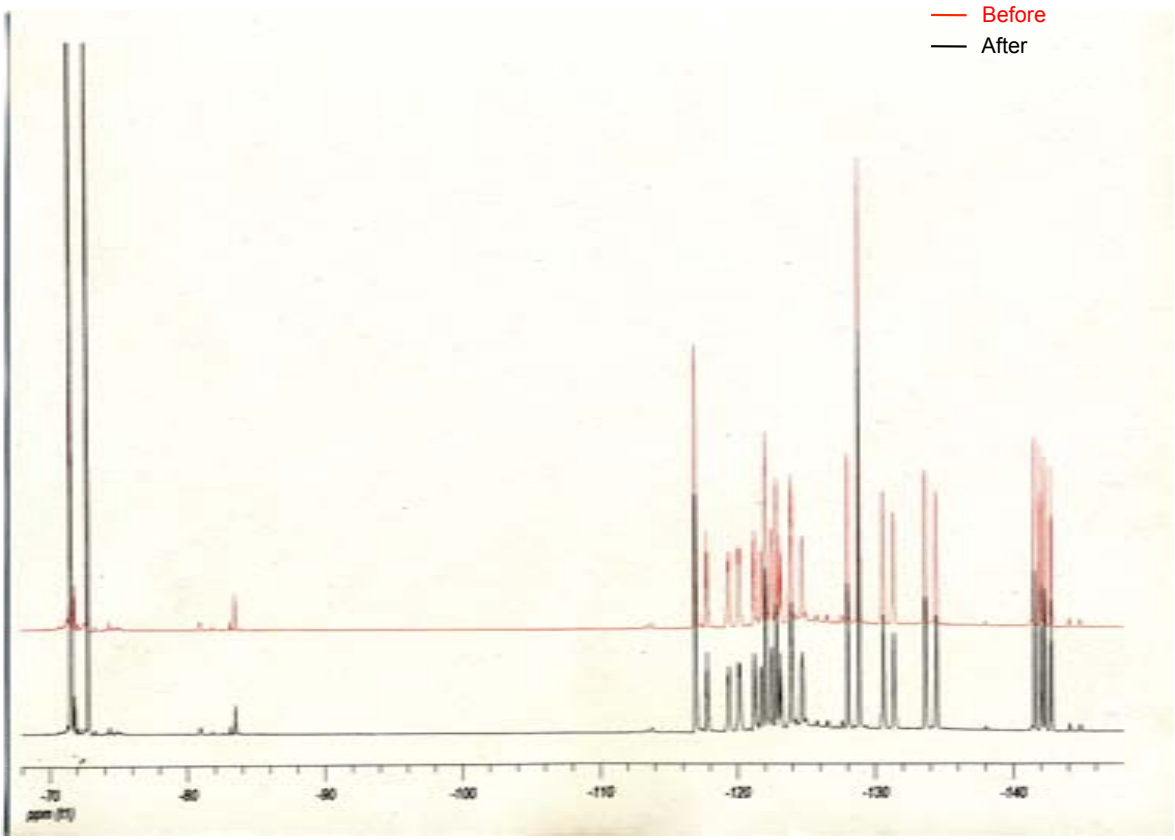

Fig. 15 Nuclear Magnetic Resonance spectroscopic analysis before (red line) and after (black line) the laser stress test. The initial absorption spectra (spikes) was coincident with the one after the laser stress test, which confirmed the stability of the blue perfluorodecalin.

6.5 Residue Test

The possible retention of dye residue inside the eye was tested by an “in vitro” and an “ex vivo” experiment. The “in vitro” experiment consisted of testing the residue formation inside a vial and the “ex vivo” experiment consisted of testing the residue retention in an enucleated pig eye, as described on section 5.3.5.

6.5.1 Residue Formation Inside the Vial

Blue PFD and blue PFnO vials were observed at two time points (7 and 14 days) and at two different temperatures (ambient and +4°C). The results obtained were the following:

- Blue PFD: Rare crystals were seen in one vial stored at +4ºC on Day 7. No crystals were detected in any other circumstance (at room temperature on day 7 or at room temperature and +4ºC on Day 14).
- Blue PFnO: Crystals were seen in all situations (ambient and +4ºC on Day 7 and Day 14) and could even be detected macroscopically on the vial walls (Fig. 16).

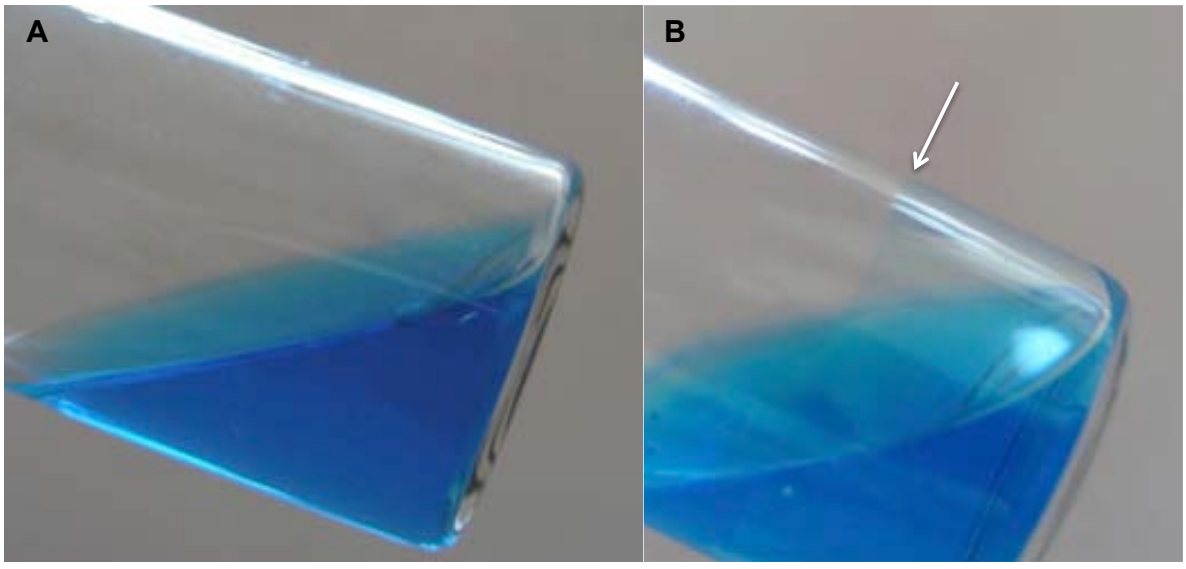

Fig. 16 Vials of **(A)** blue perfluorodecalin (PFD) and **(B)** blue perfluoro-n-octane (PFnO) stored at +4ºC for 7 days. With blue PFD **(A)** no macroscopic pigment residues were observed. With blue PFnO **(B)** pigment residues could be clearly seen staining the vial wall (white arrow).

A summary of the study results of the residue formation inside the vial test for the two types of blue colored PFCLs can be seen in Table 4.

Table 4 - Residue Formation Inside the Vial				
	Ambient Day 7	**+ 4ºC Day 7**	**Ambient Day 14**	**+ 4ºC Day 14**
Blue PFD*	No crystals	+	No crystals	No crystals
Blue PFnO†	+++	+++	+++	+++

* Blue perfluorodecalin

† Blue perfluoro-n-octane

6.5.2 Evaluation of Residual Pigment Retention Inside the Pig Eye

After vitrectomy with blue PFD, blue PFnO or blue powder injection and air exchange at the end, the enucleated pig eyes were dissected and analyzed for the presence of pigment residues in the posterior retina, residual peripheral vitreous and posterior lens capsule. The results obtained were the following:

- Blue PFD: Discrete filamentous pigment residues were seen in remaining peripheral vitreous; none on the posterior retina; none on the posterior lens capsule (Fig. 17).

- Blue PFnO: Small blue particles homogeneously localized on the whole retinal surface and residual peripheral vitreous; none on the posterior lens capsule (Fig. 17).

- Blue powder: Small blue particles heterogeneously distributed on the retinal surface and large blue particles/agglomerates detected in the peripheral vitreous samples; none on the posterior lens capsule (Fig. 17).

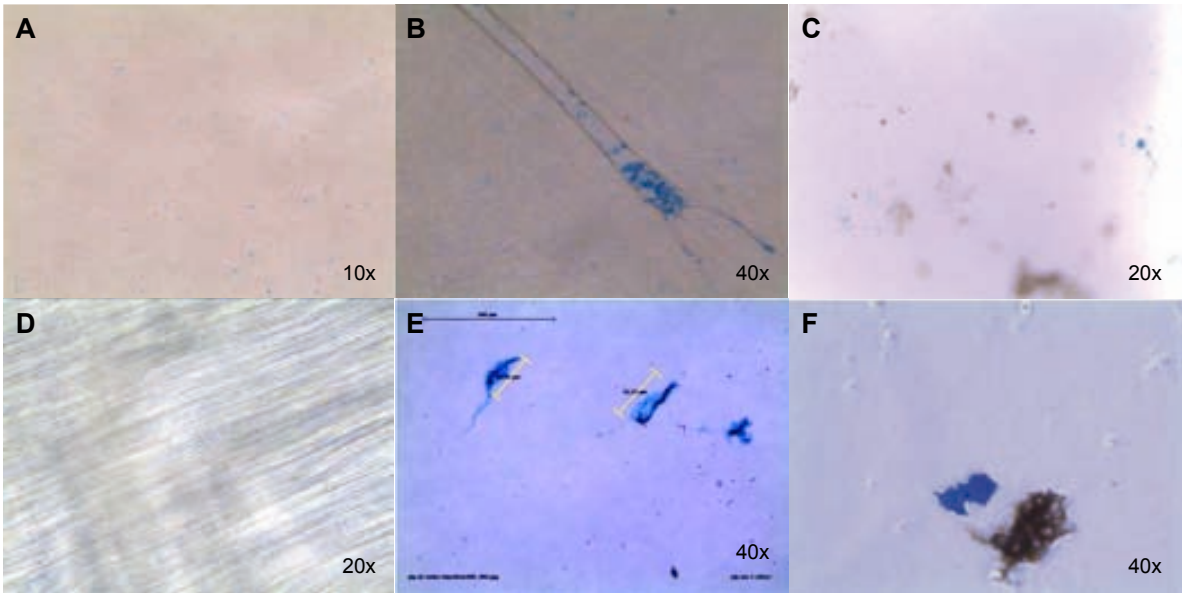

Fig. 17 (A) Pigment residues of blue perfluoro-n-octane at 10x magnification and **(B)** 40x magnification in the posterior retinal surface. **(C)** Blue powder residues in the posterior retinal surface. **(D)** Posterior lens capsule without pigment residues was observed on all groups. **(E)** Filamentous residues of blue perfluorodecalin in the residual peripheral vitreous. **(F)** Large agglomerates of blue powder in the residual vitreous.

6.6 Toxicity Test

After vitrectomy was performed on a live hybrid pig eye, three groups corresponding to different surgical procedures performed at Day 1 were established, as described on section 5.3.6.4:

- **Group 1 - Blue PFCL Toxicity**
- **Group 2 - Blue Dye Residues Toxicity**
- **Group 3 - Blue PFCL Toxicity in the Subretinal Space**

Each one of these groups were composed of three study eyes (blue PFD) and three control eyes (transparent PFnO). For this experiment and as described on section 5.2.2, an Animal Research Ethics Committee at VHIR had to be obtained.

6.6.1 Clinical Findings

All eyes were examined daily from Day 1 to Day 8.

Group 1: Between Day 3 and 5 the transparent PFnO and blue PFD underwent dispersion in all eyes (Fig. 18). A localized subcapsular opacification secondary to a lens touch during vitrectomy was observed on one eye in the control group, but did not impair the visualization of the fundus. No cellular precipitates or other signs of inflammation were observed.

Group 2: After Day 6, when the air bubble was practically reabsorbed in all cases, no blue dye residues could be observed with binocular indirect ophthalmoscopy. Additionally, no cellular precipitates or other signs of inflammation were observed.

Group 3: On all eyes the retinal detachment progressed ventrally (inferior) but not dorsally (superior), sometimes forming an inferior bullous detachment (Fig. 19). The blue PFD bubble could be seen in the subretinal space, but the bubble of transparent PFnO could not be identified by indirect binocular ophthalmoscopy. A localized subcapsular opacification secondary to a lens touch during vitrectomy was observed on one eye in the study group, but did not impair the visualization of the fundus. No cellular precipitates or other signs of inflammation was observed.

6.6.2 Electroretinographic Studies

Electroretinograms (ERGs) were performed at baseline (Day 1) and Day 8. The following results were obtained:

Group 1: A statistically significant decline of the mean *a* and *b* wave amplitudes from baseline to Day 8 was observed ($p < 0.01$; student's *T* test) in study and control eyes. This was an expected finding due to the insulating properties attributed to PFCLs in the conduction of the ERG (see Discussion). At Day 8 the *b* wave implicit times were not significantly different from those at baseline.

Group 2 and 3: No significant difference was observed from baseline to day 7 of the mean *a* and *b* wave amplitudes or the *b* wave implicit times between study and control eyes.

6.6.3 Color Fundus Photography and Fluorescein Angiography

Color fundus photography and fluorescein angiography were performed at baseline (day 0) and day 7. The following results were obtained:

Group 1: It was difficult to fully interpret the images obtained at day 7 due to the dispersion of the PFCL inside the eye (Fig. 18). The dorsal (superior) part of the image that could be observed didn't seem to show any anatomical or angiographic abnormality.

Group 2: No significant anatomical or angiographic abnormality was observed in study or control eyes.

Group 3: No significant anatomical abnormality except for the expected ventral (inferior) retinal detachment (Fig. 19). Images of the inferior retina were difficult to obtain due to the lack of mobilization of the pig eye. No angiographic abnormality was observed in study or control eyes.

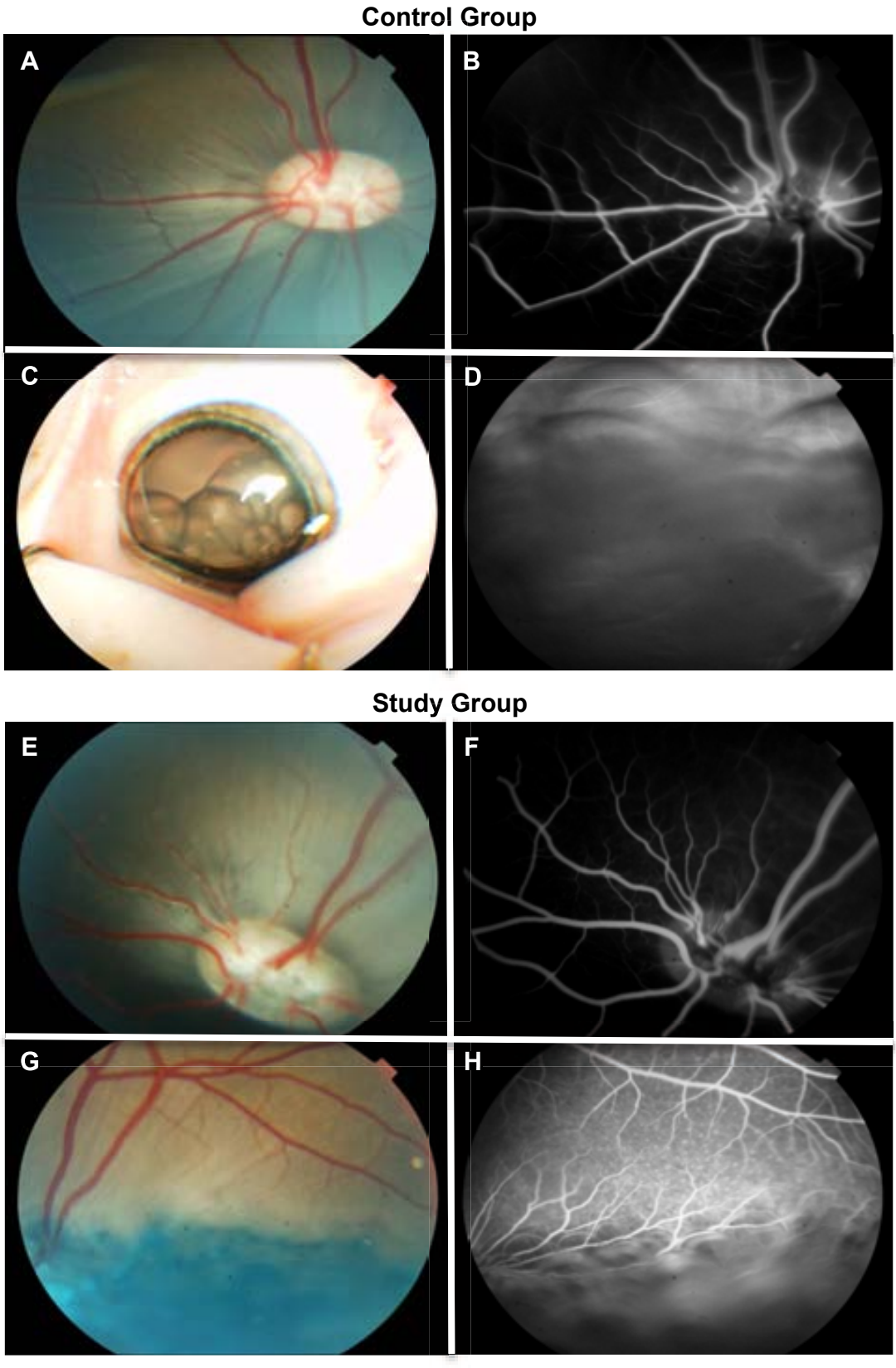

Fig. 18 Images from **(A)** to **(D)** are from the control group 1 (transparent perfluoro-n-octane) and from **E** to **H** are from the study group 1 (blue perfluorodecalin). **(A)** and **(E)** are the retinographies obtained at baseline. **(B)** and **(F)** are the angiographies obtained at baseline. **(C)** and **(G)** are the retinographies obtained at day 7. **(D)** and **(H)** are the angiographies obtained at day 7. Dispersion of the PFCL in the control group [**(C)** and **(D)**] and in the study group [**(G)** and **(H)**] impairing the correct interpretation of the images obtained can be observed.

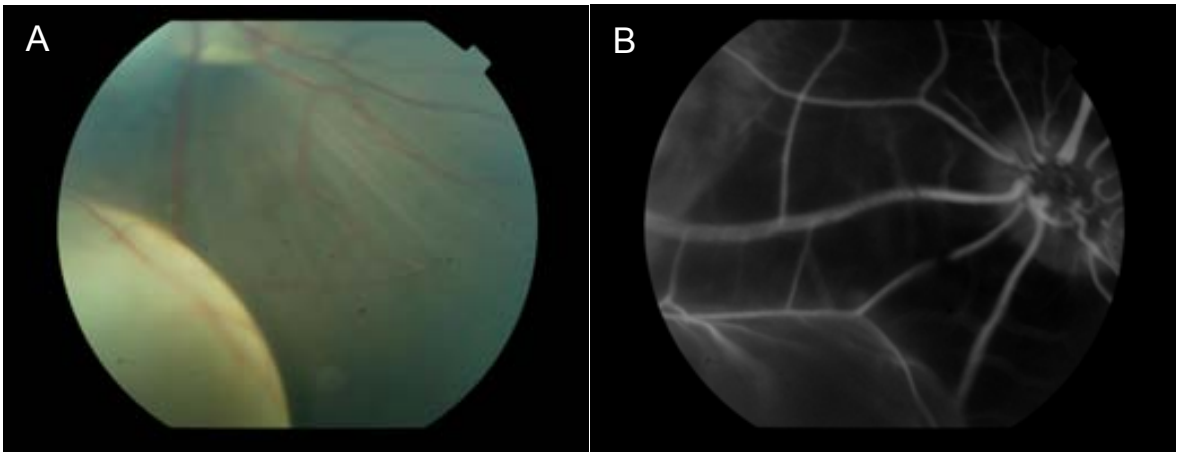

Fig. 19 (A) Ventral (inferior) bullous retinal detachment produced by progression of the original detachment. **(B)** Fluorescein angiography near an area with retinal detachment. Same eye seen in **(A)** but in a dorsal (superior) area. No angiographic changes could be seen in or around the area of retinal detachment.

6.6.4 Histologic Studies

After being fixed in 10% formaldehyde for at least 24h at room temperature and coated with paraffin, 3 µm sequential cuts were made and 10 sections per eye were obtained and stained with hematoxylin-eosin, as described on section 5.3.6.4. The following parameters were evaluated:

6.6.4.1 Retinal integrity and morphological abnormalities:

Group 1: After the globe was opened, in the study group (blue PFD) no macroscopic or microscopic staining of the retina or posterior lens capsule could be observed. No morphological abnormalities were detected (Fig. 20). The retina was detached in several cuts due to fixation artifacts.

Group 2: After the globe was opened, in the study group (blue PFD) no macroscopic or microscopic pigment residues could be observed in the vitreous cavity. No morphological abnormalities were detected (Fig. 20). The retina was detached in several cuts due to fixation artifacts.

Group 3: No morphological abnormalities adjacent to the detached retina were detected (Fig. 20). In the areas where the retina was detached and in contact with the PFCL there was swelling of all layers and vacuolization of the nuclear layers with an increase in piknotic nuclei.

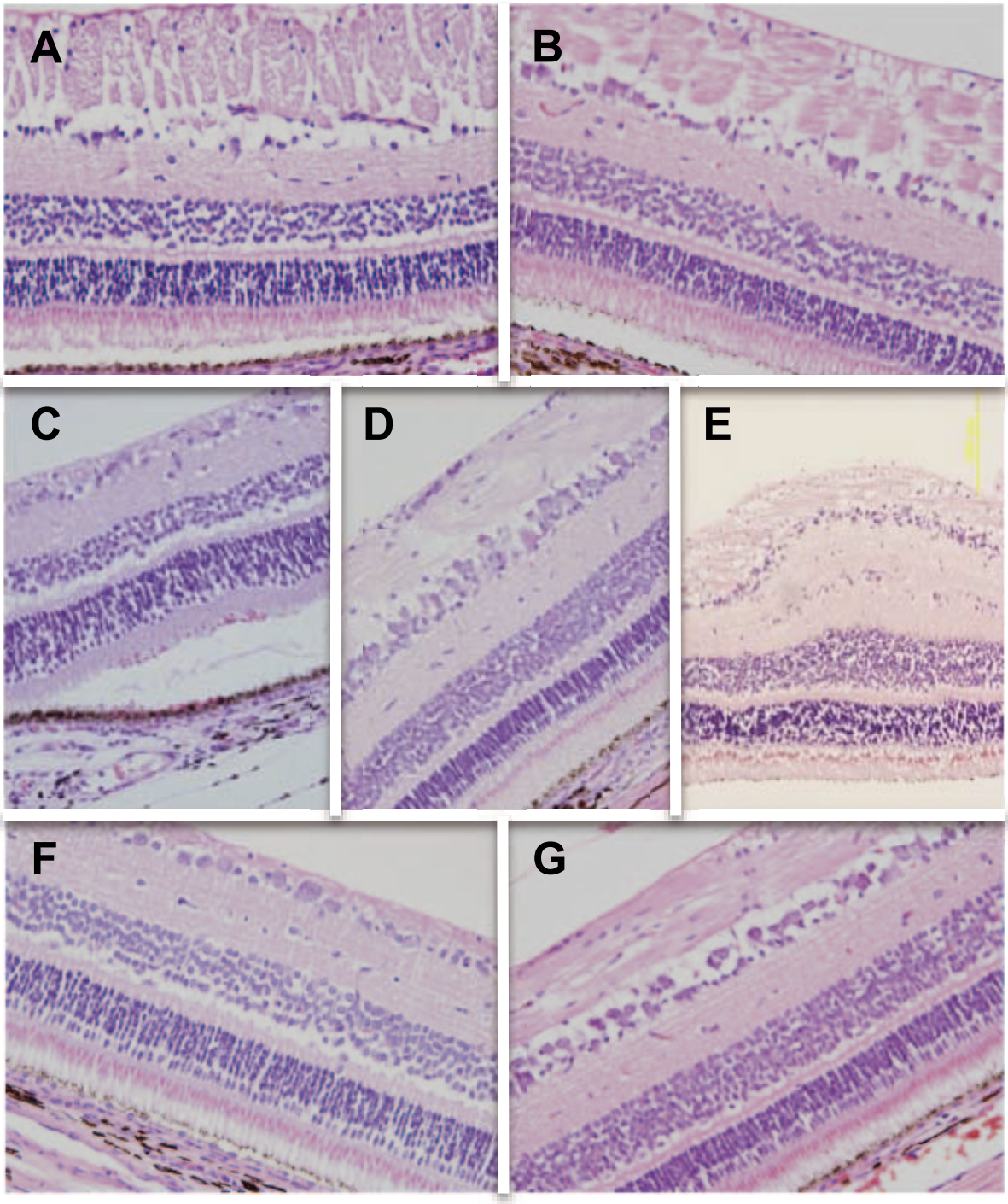

Fig. 20 Histologic images of group 1 [**(A)** and **(B)**], group 2 [**(C)** and **(D)**] and group 3 [**(E)**, **(F)** and **(G)**]. **(A)**, **(C)** and **(F)** correspond to hystologic images of the control group (perfluoro-n-octane) and **(B)**, **(D)**, **(E)** and **(G)** of the study group (blue perfluorodecalin). No morphologic abnormalities could be observed in these hematoxylin-eosin stained samples. In several of these cuts [**(A)**, **(B)** and **(C)**] a neurosensorial retinal detachment secondary to fixation artifacts can be observed. Retinal edema and vacuolization of the inner and outer nuclear layers with an increase in piknotic nuclei **(E)** could be observed on group 3 in the area where the detached retina was in contact with the PFCL.

6.6.4.2 Retinal ganglion cell number:

Group 1, 2 and 3: No significant difference between the control (transparent PFnO) and study (blue PFD) eyes on all 3 groups was found in the mean total ganglion cell number or the percentage of live ganglion cells (tables 5, 6 and 7).

Table 5. Group 1 Mean Ganglion Cell Count Per Field

	Central Retina				Peripheral Retina			
Animal/ Eye	Live	Dead	Total	% Live	Live	Dead	Total	% Live
32 OD*	26,30	2,50	28,80	89,55	21,40	2,90	24,30	85,75
33 OD*	30,70	2,40	33,10	92,75	22,00	3,10	25,10	83,42
34 OD*	17,90	3,20	21,10	84,83	19,70	3,60	23,30	84,55
Mean*	24,97	2,70	**27,67**	**90,24**	21,03	3,20	**24,23**	**86,79**
41 OD†	34,10	3,60	37,70	90,45	25,20	2,00	27,20	92,65
44 OD†	24,30	1,40	25,70	94,55	23,10	1,50	24,60	93,90
80 OD†	33,20	2,00	35,20	94,32	15,90	1,60	17,50	90,86
Mean†	30,53	2,33	**32,87**	**92,88**	21,40	1,70	**23,10**	**92,64**

* Control Group with Transparent Perfluoro-n-Octane
† Study Group with Blue Perfluorodecalin

Table 6. Group 2 Mean Ganglion Cell Count Per Field								
	Central Retina				Peripheral Retina			
Animal/ Eye	Live	Dead	Total	% Live	Live	Dead	Total	% Live
36 OD*	26,70	3,20	29,90	89,30	18,60	3,10	21,70	85,71
38 OD*	22,22	3,44	25,67	86,58	22,56	3,00	25,56	88,26
82 OD*	23,40	3,40	26,80	87,31	22,56	2,56	25,11	89,82
Mean*	24,11	3,35	**27,46**	**87,81**	21,24	2,89	**24,12**	**88,04**
45 OD†	22,90	2,40	25,30	90,51	20,10	2,30	22,40	89,73
83 OD†	27,44	2,22	29,67	92,51	17,44	3,11	20,56	84,86
84 OD†	21,40	3,10	24,50	87,35	15,80	2,20	18,00	87,78
Mean†	23,91	2,57	**26,49**	**90,28**	17,78	2,54	**20,32**	**87,51**

* Control Group with Transparent Perfluoro-n-Octane

† Study Group with Blue Perfluorodecalin

Table 7. Group 3 Mean Ganglion Cell Count Per Field								
	Central Retina				Peripheral Retina			
Animal/ Eye	Live	Dead	Total	% Live	Live	Dead	Total	% Live
35 OD*	22,20	5,30	27,50	80,73	17,40	4,30	21,70	80,18
40 OD*	25,33	2,67	28,00	90,48	19,44	2,11	21,56	90,21
81 OD*	24,30	3,40	27,70	87,73	16,90	2,60	19,50	86,67
Mean*	23,94	3,79	**27,73**	**86,34**	17,91	3,00	**20,92**	**85,64**
42 OD†	22,50	2,10	24,60	91,46	22,10	1,90	24,00	92,08
47 OD†	17,89	2,33	20,22	88,46	15,22	2,44	17,67	86,16
88 OS†	24,50	3,00	27,50	89,09	15,70	2,70	18,40	85,33
Mean†	21,63	2,48	**24,11**	**89,72**	17,67	2,35	**20,02**	**88,27**

* Control Group with Transparent Perfluoro-n-Octane

† Study Group with Blue Perfluorodecalin

6.6.4.3 Correlation of the thickness of several retinal layers:

Correlation of thickness for each group was obtained by comparing the measurements of the mean retinal layer thickness [ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and photoreceptor layer (PRL)] and of mean total retinal thickness of all control eyes (PFnO) with the same measurements made in the study eyes (blue PFD). The retinal nerve fiber layer (RNFL) was not part of the comparison due to differences greater than 100 μm between the study or control eyes, a difference greater than of the other layers. When compared to the fellow eye the RNFL presented similar thickness. The total retinal thickness measured in the fellow eyes ranged from 350 μm to 450 μm.

When comparing retinal thicknesses between groups, the following results were obtained:

Group 1, 2 and 3: A statistically significant difference of the mean total retinal thickness between control and study eyes was observed on all groups ($p < 0.05$; student's *T* test). The control eyes had a mean total retinal thickness superior to the study eyes in groups 1 and 3, and in the posterior retina of group 2. But when each pig eye was compared to the fellow eye, this difference was insignificant (see Discussion). No appreciable difference between control and study eyes was observed when individual layer thickness was analyzed (Fig. 21, Fig. 22 and Fig. 23).

6.6.4.4 Apoptosis:

Apoptosis analysis was performed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Group 1, 2 and 3: The TUNEL assay was negative on all samples, meaning that absence of apoptosis was observed on the control (transparent PFnO) and study (blue PFD) eyes of all 3 groups.

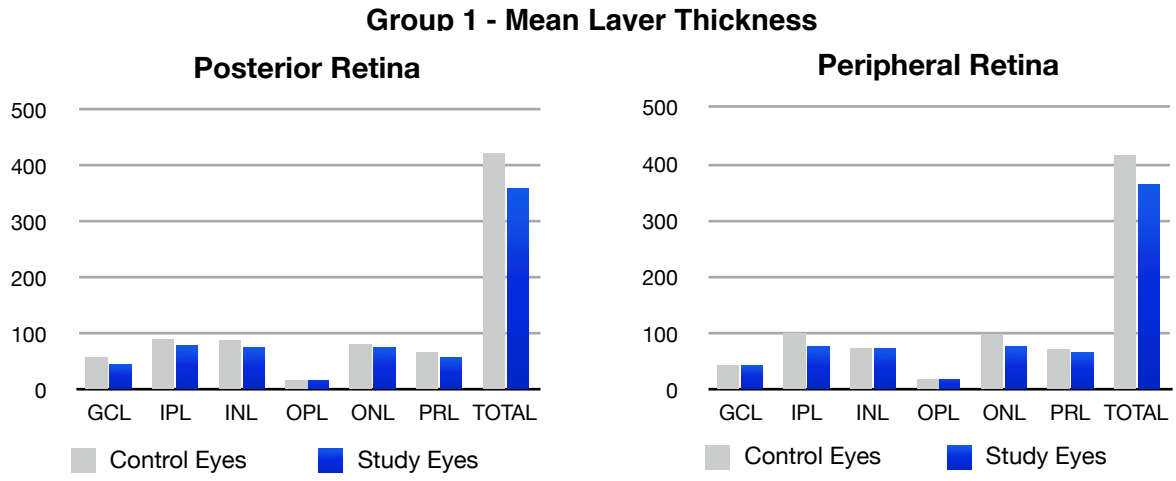

Fig. 21 Graphics showing the posterior and peripheral retinal thickness measurements made on the control and study eyes of group 1 subjects. The mean total retinal thickness difference between control and study eyes was significant either in the posterior or peripheral retina ($p < 0.05$; student's *T* test).

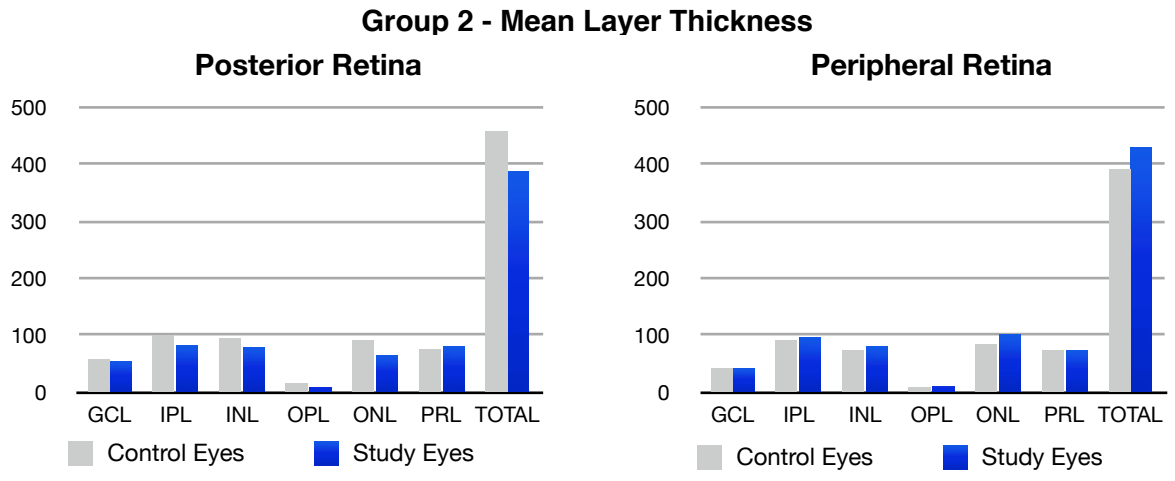

Fig. 22 Graphics showing the posterior and peripheral retinal thickness measurements made on the control and study eyes of group 2 subjects. The mean total retinal thickness difference between control and study eyes was significant either in the posterior or peripheral retina ($p < 0.05$; student's *T* test).

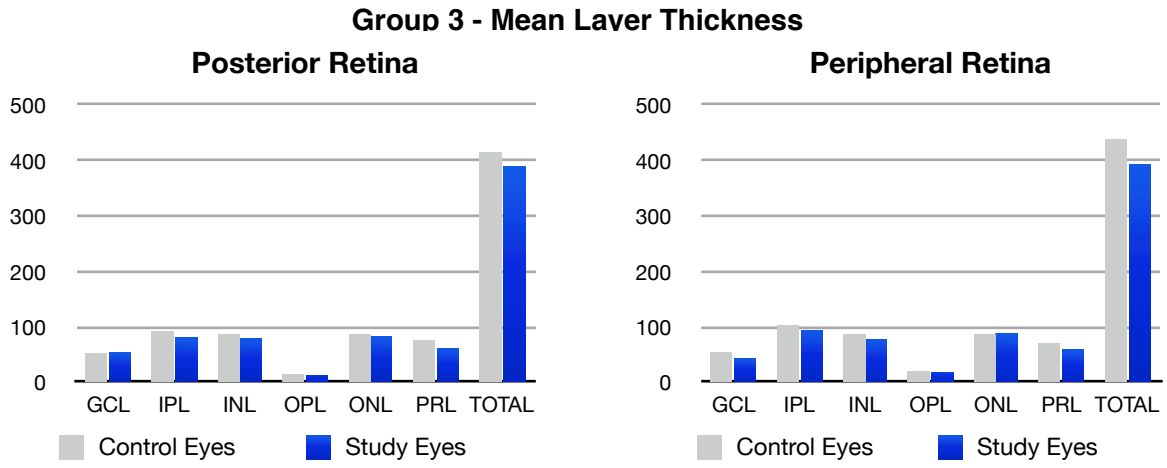

Fig. 23 Graphics showing the posterior and peripheral retinal thickness measurements made on the control and study eyes of group 3 subjects. The mean total retinal thickness difference between control and study eyes was significant either in the posterior or peripheral retina ($p < 0.05$; student's *T* test).

7. DISCUSSION

7. DISCUSSION

7.1 Our Study

To evaluate the potential of colored perfluorocarbon liquids (PFCLs) in vitreoretinal surgery, it was first ascertained for optimal concentration followed by intra-operative behavior in the enucleated pig eye. Three other important issues were also addressed by different “in vitro” and “ex vivo” experiments: (1) silicone oil miscibility of the colored PFCLs at our disposal; (2) stability of those colored PFCLs after endolaser photocoagulation; (3) retention of dye residue after removal of the colored PFCLs. Additionally, toxicity in the live pig eye was verified.

7.1.1 Surgical Behavior and Other Colored PFCL Related Issues

The color of the PFCLs that is seen on the images used in this research project was obtained after filtration with a 0.22 μm polytetrafluoroethylene (PTFE) filter. It is important to bear in mind that if the PFCL is not filtered its color will be different, since a portion of the dye is trapped in the filter. This was most evident in PFCLs that are directly stained [blue perfluorodecalin (PFD) or blue perfluoro-n-octane (PFnO)]. So even if filtration for this kind of medical devices is not essential due to their inert properties, to obtain the same color it is necessary to filter the colored PFCL. Transparency could also be affected if the colored PFCL is not filtered, as the filtration process reduces the dye that is in excess.

During intra-operative behavior and silicone oil miscibility tests, it became evident the superiority of the directly colored PFCLs over the others that were a mixture with a semifluorinated alkane (SFA). As SFAs, like perfluorohexyloctane (F6H8), are used to make heavier-than-water silicone oils, it would be expected that those PFCLs that are mixed with a colored SFA were miscible with silicone oil during direct exchanges, like it was observed in our study. At issue is when the PFCL vaporizes under an air exchange and residual SFA could be left inside the eye. As discussed in more detail in section 7.2, this could increase silicone oil

emulsification rate or raise inflammatory and even toxicity concerns and for that reason they were excluded from further testing. The main advantage of the directly colored PFCLs is that the dye dissolves predictably inside the PFCL limiting its diffusion in the eye and contact with the retina. Although pigment residues were identified with these compounds after air exchanges, they were only identified in the enucleated pig eye experiment and did not show up at Day 8 of the toxicity evaluation. Further, they did not produce inflammation or direct toxicity in retinal cells, suggesting that they might be eliminated by the organism. The directly colored blue PFnO and PFD were unique in that they provided a clear view during air exchanges and in the subretinal space while maintaining transparency to work on the retinal surface and immiscibility with silicone oil.

Subretinal visualization is particularly important, as a recent study shown that with the microincision techniques (23G and 25G) subretinal retention of these compounds might be increasing.[131] Normally large bubbles of PFCL that migrate to the subretinal space can be distinguished from the subretinal fluid by their rounder form. But the problem arises when small bubbles migrate to the macular area through the peripheral tears. In this situation and with the retina detached it is difficult to identify these bubbles due to their transparent nature. Blue PFD could reduce this important complication by allowing intra-operative visualization and removal of these small bubbles from the macular area, as described by some authors post-operatively.[132,133]

PFCLs have been shown to have high transparency to light in the visible spectrum. They present no obstacle to laser photocoagulation in the visible and infrared spectrum, so initial laser power settings should be kept low because the transmission of the PFCLs is superior to that of balanced salt solution.[153] Blue PFnO and blue PFD would be expected to impair retinal photocoagulation as their absorbance spectrum could affect the uptake of the energy by the retinal pigment epithelium (RPE) cells. In our study we performed diode laser photocoagulation in continuous and pulsed mode and observed that exposure time or potency should be slightly increased but that didn't affect the intensity of the laser burn nor resulted in significant time consumption. Equally important was the fact that stability of these compounds was not altered after intense photocoagulation.

7.1.2 Toxicity Testing

Most of the published articles of PFCL tolerance in animals use rabbit eyes in their research.[97,106,107,109,110,112,154] But because the rabbit retina is thin and avascular,[155] it may be more susceptible to mechanical factors than the pig eye.[152] In recent years it appears to have been an increase in literature about the anatomy and physiology of the pig eye because of an expansion in its use as a model for research. Pig eyes share many similarities with human eyes, having a holangiotic retinal vasculature, no tapetum, cone photoreceptors in the outer retina, and a similar scleral thickness, rendering them valuable in comparative research.[156] For these reasons, this study followed the preference for pig eyes. The sample size for the “in vivo” experiments was of 3 pig eyes per group, as it was the smallest necessary to evaluate the hypotheses formulated with minimal errors (see section 7.3). The follow-up period was of 7 days, because, in the majority of published research articles on PFCL retinal tolerance,[97,107] after this period some changes start to occur in the retina that could lead to a misinterpretation of the results (see section 7.3).

In the group with blue PFD, a significant reduction of the ERG amplitudes was noted at Day 8. This decrease is probably due to an insulating effect of the PFD in the electric conductivity of the ERG. Similar insulating effects have been described with other PFCLs[97,154] and with silicone oil.[157-159] Studies with silicone oil have shown that the extent of the insulating effect is related to the degree of fill.[159] Recovery of the *a* and *b* wave amplitudes normally occurs after the 5th day of removal.[97] It doesn't occur earlier due to the probable operative trauma that can strongly affect the amplitude of the corneal ERG,[160] and this was the reason why we didn’t performed an ERG before Day 8. The fact that in our study the groups with BSS didn’t show a reduction of the amplitudes of the ERG and that no histologic alteration was found, indicates that the this decrease may be due to the described insulating effect of the blue PFD and transparent PFnO, rather than a toxic effect.

White flake-like precipitates have been described in the interface of the perfluorochemical and the remaining vitreous.[107] In our study no such finding was

observed. This might be due to the fact that in our study we performed a posterior hyaloid peeling and peripheral vitrectomy, leaving only a small amount of vitreous in the eye, whereas in the previous article no hyaloid peeling or peripheral vitrectomy were performed, leaving a considerable amount of vitreous in the eye.

Dispersion of perfluorocarbon liquids has been reported to occur in the first week after injection in rabbit eyes.[97,107] It is recognized by surgeons and manufacturers that PFD is less prone to disperse than PFnO. In our study dispersion of PFnO and blue PFD occurred between Day 3 and Day 5, and no significant difference in dispersion was seen between the two PFCLs. Although unlikely, this might be due to the fact that the PFD is colored by the blue dye. The dispersion of these heavy liquids affected the visualization of the fundus, and interpretation of retinography and angiography in the group of eyes that were filled with PFCL. In the groups were the eye was filled with BSS, no anatomical or angiographic alteration was detected.

We analyzed the eighteen study and control eyes and the eighteen fellow eyes histologically to observe changes in retinal anatomy or other morphologic abnormalities. Chang et al.[97] reported occasional macrophages containing oil-like vacuoles on the inner retinal surface and “photoreceptor drop-down” (displacement of photoreceptor nuclei into the rod and cone layer) in the inferior retina at 1 week with PFnO. Eckardt et al.[107] that also studied tolerance to PFnO reported hypertrophy and bumplike protrusions of Müller cells, macrophages in the inner plexiform layer and irregularities of the outer segments by day 6. On these reports rabbit eyes were used. In our study, none of these changes were observed and this might be related to the greater retinal thickness of the pig eye compared to the rabbit eye, as described above.

Retinal ganglion cells (RGC) count was also performed in our study. Numerous neurodegenerative diseases including retinal ischemia, diabetic retinopathy and glaucoma adversely affect RGC survival.[161] RGC are regularly used for “in vitro”[162] and “in vivo”[163] dye toxicity analysis. In our study no RGC count differences were found between study and control eyes.

To our knowledge there are no normal intervals of retinal thickness described for the hybrid pig eye used in our “in vivo” experiments, but the fellow eyes in our study had a total retinal thickness that ranged from approximately 350 μm to 450 μm. Because differences in mean total retinal thickness were obtained between control and study eyes, each control and study eye was compared to its fellow eye to establish if the differences observed were due inter-individual changes. As no significant changes were found between both eyes of the same animal, the differences obtained were interpreted as inter-individual changes.

In our study apoptotic analysis was performed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and was considered negative. TUNEL staining has nearly universally been adopted as the method of choice for detecting apoptosis *in situ*. TUNEL staining may also be used to detect DNA damage associated with non-apoptotic events such as necrotic cell death induced by exposure to toxic compounds and other contaminants,[164] and TUNEL staining has also been reported to stain cells undergoing active DNA repair.[165,166] Therefore TUNEL staining may be considered generally as a method for the detection of DNA damage (DNA fragmentation), and under the appropriate circumstances, more specifically as a method for identifying apoptotic cells.

7.2 Review of the Literature

To our knowledge the only PubMed available data on colored perfluorocarbon liquids was published by Rizzo et al.[146] In his work he uses a colored semifluorinated alkane (F_6H_8) to color the PFnO or the PFD. The colored PFCLs were studied “in vitro” (in the vials) and “ex vivo” (in enucleated pig eyes). The “in vitro” cytotoxicity according to the ISO 10993-5 standard by Bioservice Scientific Laboratories (Planegg/Munich, Germany) on all tested concentrations showed no significant growth inhibition. They also found that colored PFCLs were clearly visible and allowed for underlying retina visualization. A time-dependent diffusion of the dye into the silicone oil was observed and they suggest that contact

between silicone oil and their colored PFCL should be limited. This was found either “in vitro” and “ex vivo”.

As reported on sections 6.2.4 and 6.3, our group has also found the same results with these type of colored PFCLs, which are a mixture of a PFCL with a SFA, and showed that they are clearly inferior to the directly colored blue PFD used in our study. These mixed colored compounds (PFCL + colored SFA) were excluded from our study during its first phase due to concerns related to the retention of colored SFAs after direct silicone oil - colored PFCL exchange or even in air exchanges. If present inside the eye, the perfluorohexyloctane (F_6H_8) has been reported to produce significant intraocular inflammation, epiretinal membrane formation, retrolental proliferation, cystoid macular edema[167,168] and even toxicity. [169] Furthermore if mixed with regular silicone oil it can increase its emulsification rate.[170,171] In our opinion the colored PFCL used by Rizzo et al.[146] should not be used in clinical practice due to the concerns related with the colored perfluorohexyloctane retention.

Although Rizzo et al. conclude their preclinical work[146] by saying that further studies should be performed to evaluate the feasibility of the colored PFCLs in human eyes, about 14 months previous to this work, Rizzo published an opinion article in Retina Today magazine (March 2011 edition)[172] where he had already performed human testing. In this initial clinical testing (pilot study), which involved 10 patients, air-colored PFCL and direct silicone oil-colored PFCL exchanges were done, even though time-dependent diffusion of the dye into the silicone oil was observed on their preclinical study. He found that colored PFCLs could facilitate safe and efficient removal of temporary tamponades on human subjects.

7.3 Limitations and Future Applications

The two major limitations of this study relate to the sample size and duration of follow-up in the “in vivo” toxicity study performed in the live hybrid pig eye.

As described above the sample size for the “in vivo” experiments was of 3 pig eyes per group. Since most results were consistent in each group, increasing the sample size by a few eyes wouldn’t improve greatly the confidence of the results. To improve the level of confidence to 90% and reduce the margin of error to bellow 10%, the sample size would have to be of approximately 70 eyes per group. This would imply having 210 pig eyes in the study group, which would render this study unfeasible. For this reason in our study we used the smallest sample size to evaluate the hypotheses formulated with minimal errors, a tendency observed in some landmark studies.[97,107]

The follow-up period was of 7 days. It would be expected that if the dye were to be toxic, it would produce at least some inflammation, electroretinogram (ERG) changes, alteration of the retinal vascular permeability, histologic changes or apoptosis by Day 8. As none of these were observed, the follow-up period was not extended. Additionally, the majority of published research on PFCL retinal tolerance,[97,107] show that some changes start to occur in the retina after one week, which could lead to a misinterpretation of the results.

The perfluorocarbon liquids (PFCLs) characteristics allow them to be widely and successfully used in the management of several vitreoretinal diseases. As experience with PFCLs continues to grow, new applications for their use may arise.

The colored PFCLs appear to offer a surgical advantage in certain steps of vitreoretinal surgery. The blue PFD clearly facilitates the view during the exchanges and in the subretinal space and generally provides a better visualization. Due to it’s characteristics, blue PFD could also help to reduce intraocular retention rates observed with the transparent PFCLs. It may be useful in teaching residents and fellows, and even experienced surgeons could benefit with improved identification of the PFCL interface in patients with cloudy corneas or lens opacification.

8. CONCLUSION

8. CONCLUSION

1. Red, yellow and blue colored perfluorocarbon liquids (PFCLs) provide an improved boundary visualization under air exchange compared to transparent perfluoro-n-octane (PFnO).

2. Directly colored [blue PFnO and blue perfluorodecalin (PFD)] improve visualization under silicone oil exchanges and and appear to be immiscible, a problem inherent to semi-fluorinated alkane-based colored PFCLs (red and yellow PFCLs).

3. Blue colored PFCLs provide a better visualization in the subretinal space compared to yellow or transparent PFCLs.

4. Blue PFD and blue PFnO don't appear to degrade laser application when applied to the retina and provide stability after intense laser photocoagulation.

5. Blue PFD doesn't leave significant pigment residues in the vitreous cavity, unlike blue PFnO.

6. Blue PFD was found to be non-toxic according to the "in vitro" cytotoxicity tests (ISO 10993-5:2009 on BalbC 3T3 cell culture) and the "in vivo" pig eye tests performed.

In conclusion, blue PFD was found to have the preferred characteristics to achieve the objective of reducing complications typically observed with currently used transparent PFCLs. To further investigate this conclusion and promote the use of directly-colored PCFLs, a formal Phase 1 study should be performed.

9. BIBLIOGRAPHY

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1. Clark LC, Gollan F. Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. Science. 1966;152:1755-1756.

2. Haidt SJ, Clark LC Jr, Ginsberg J. Liquid perfluorocarbon replacement of the eye (abstract). Invest Ophthalmol Vis Sci. 1982;22(Suppl):233.

3. Clark LC. Description, U.S. Patent no. 4,490,351. 1984.

4. Zimmerman NJ, Faris D. The use of N-perfluorocarbon amines in complicated retinal detachments (abstract). Invest Ophthalmol Vis Sci. 1984;25(Suppl): 258

5. Chang S. Low viscosity liquid fluorochemicals in vitreous surgery. Am J Ophthalmol. 1987;103:38-43.

6. Chang S, Ozmert E, Zimmerman NJ. Intraoperative perfluorocarbon liquids in the management of proliferative vitreoretinopathy. Am J Ophthalmol. 1988;106(6):668-674.

7. Chang S, Reppucci V, Zimmerman NJ, et al. Perfluorocarbon liquids in the management of traumatic retinal detachments. Ophthalmology. 1989;96(6): 785-91; discussion 791-2.

8. Brazitikos PD, Androudi S, Dimitrakos SA, Stangos NT. Removal of the internal limiting membrane under perfluorocarbon liquid to treat macular-hole-associated retinal detachment. Am J Ophthalmol. 2003;135(6):894-6.

9. Pon DM. Chemical characteristics of perfluorocarbon liquids and silicone oil for complex retinal detachments. Vitreoretinal Surg Technol. 1990;2(1):6.

10. Friberg TR, Tano Y, Machemer R. Streaks (schlieren) as a sign of rhegmatogenous detachment in vitreous surgery. Am J Ophthalmol. 1979;88(5):943-4.

11. Wong R. Gupta B, Aylward GW, Laidlaw DA. Dye extrusion technique (DE-TECH): occult retinal break detection with subretinal dye extrusion during vitrectomy for retinal detachment repair. Retina. 2009;29(4):492-6.

12. Larrison WI, Frederick AR, Peterson TJ, Topping TM. Posterior retinal folds following vitreoretinal surgery. Arch Ophthalmol. 1993;111(5):621-625.

13. Heimann H, Bopp S. Retinal folds following retinal detachment surgery. Ophthalmologica. 2011;226(suppl 1):18–26.

14. dell'Omo R, Tan HS, Schlingemann RO, et al. Evolution of outer retinal folds occurring after vitrectomy for retinal detachment repair. Invest Ophthalmol Vis Sci. 2012;53(13):7928-35.

15. Carroll BF, Peyman GA, Mehta NJ, et al. Repair of retinal detachment associated with proliferative vitreoretinopathy using perfluoroperhydrophenanthrene (Vitreon). Vitreon Study Group. Can J Ophthalmol. 1994;29:66-69.

16. Coll GE, Chang S, Sun J, et al. Perfluorocarbon liquid in the management of retinal detachment with proliferative vitreoretinopathy. Ophthalmology. 1995;102:630-638.

17. Han DP, Rychwalski PJ, Mieler WF, et al. Management of complex retinal detachment with combined relaxing retinotomy and intravitreal perfluoro-n-octane injection. Am J Ophthalmol. 1994;118(1):24-32.

18. Forlini C. centrifugal surgical strategy with early introduction of PFCL in the management of retinal tractional detachment: Effect on Reproliferation. J Vitreo Retina. 1992;1:44-51.

19. Forlini C. Evaluation of the use of heavy liquids in vitreoretinal surgery: Personal Experiences. Vitreoretinal Surgical Technol. 1993;5:6-8.

20. Schulman JA, Peyman GA, Blinder KJ, et al. Management of giant retinal tears with perfluoroperhydrophenanthrene (Vitreon). Jpn J Ophthalmol. 1993;37(1):70-7.

21. Forlini C, Del Fiume E, Cicognani A, D'Eliseo D. Use of PFCL in the surgical management of endophthalmitis: New indicators. J Vitreo Retina. 1992;1:55-63.

22. Machemer R. Retinotomy. Am J Ophthalmol. 1981;92(6):768-74.

23. Haut J, Larricart P, van Effenterre G. Localized retinectomy indications in the treatment and prevention of retinal detachment. Ophthalmologica. 1984;188(4):212-5.

24. Morse LS, McCuen BW 2nd, Machemer R. Relaxing retinotomies. Analysis of anatomic and visual results. Ophthalmology. 1990;97(5):642-7; discussion 647-8.

25. Ando F, Kondo J. A plastic tack for the treatment of retinal detachment with giant tear. Am J Ophthalmol. 1983;95(2):260-1.

26. de Juan E Jr, Hickingbotham D, Machemer R. Retinal tacks. Am J Ophthalmol. 1985;99(3):272-4.

27. Ando F, Kondo J. Surgical techniques for giant retinal tears with retinal tacks. Ophthalmic Surg. 1986;17(7):408-11.

28. Abrams GW, Williams GA, Neuwirth J, McDonald HR. Clinical results of titanium retinal tacks with pneumatic insertion. Am J Ophthalmol. 1986;102(1):13-9.

29. Glaser BM, Carter JB, Kuppermann BD, Michels RG. Perfluoro-octane in the treatment of giant retinal tears with proliferative vitreoretinopathy. Ophthalmology. 1991;98(11):1613-21.

30. Quiroz-Mercado H, Guerrero-Naranjo J, Agurto-Rivera R, et al. Perfluorocarbon-perfused vitrectomy: A new method for vitrectomy - A safety and feasibility study. Graefes Arch Clin Exp Ophthalmol 2005;243:551-262.

31. Arevalo JF. En bloc perfluorodissection in vitreoretinal surgery: A new surgical technique. Retina. 2008;28(4):653-6.

32. Dalma-Weiszhausz J, Franco-Cardenas V, Dalma A. Subretinal perfluorocarbon liquid for dissection of proliferative vitreoretinopathy. J Ophthalmic Vis Res. 2012;7(4):350-4.

33. de Juan E Jr, McCuen B, Tiedeman J. Intraocular tamponade and surface tension. Surv Ophthalmol. 1985;30(1):47-51.

34. Michels RG, Rice TA, Blankenship G. Surgical techniques for selected giant retinal tears. Retina. 1983;3(3):139-53.

35. Leaver PK, Cooling RJ, Feretis EB, et al. Vitrectomy and fluid/silicone-oil exchange for giant retinal tears: Results at Six Months. Br J Ophthalmol. 1984;68(6):432-8.

36. Peyman GA, Smith RT. Use of fluorosilicone to unfold a giant retinal tear. Int Ophthalmol. 1987;10(3):149-51.

37. Brown GC, Benson WE. Use of sodium hyaluronate for the repair of giant retinal tears. Arch Ophthalmol. 1989;107(8):1246-9.

38. Chang S, Lincoff H, Zimmerman NJ, Fuchs W. Giant retinal tears. Surgical techniques and results using perfluorocarbon liquids. Arch Ophthalmol. 1989;107(5):761-6.

39. Chang S, Kwun RC: Perfluorocarbon liquids in vitreoretinal surgery. In Retina (4th Edition), SJ Ryan (Ed.). Philadelphia, PA: Elsevier Mosby, 2006, pp. 2179-2190

40. Kreiger AR, Lewis H. Management of giant retinal tears without scleral buckling. Use of radical dissection of the vitreous base and perfluoro-octane and intraocular tamponade. Ophthalmology. 1992;99(4):491-7.

41. Ambresin A, Wolfensberger TJ, Bovey EH. Management of giant retinal tears with vitrectomy, internal tamponade, and peripheral 360 degrees retinal photocoagulation. Retina. 2003;23(5):622-8.

42. Desai UR, Peyman GA, Harper CA III. Perfluorocarbon liquids in traumatic vitreous hemorrhage and retinal detachment. Ophthalmic Surg. 1993;24:537-541.

43. Resnick KJ, Shapiro MJ, Kim S. Perfluorooctane as an instrument to manipulate intraocular foreign bodies (abstract). Invest Ophthalmol Vis Sci. 1991;32(suppl):881.

44. Sudhalkar HA, Johnson MW. Perfluorocarbon liquid manipulation of high-density intraocular foreign bodies. Retina. 1998;18(5):460-5.

45. Han DP, Mieler WF, Abrams GW, Williams GA. Vitrectomy for traumatic retinal incarceration. Arch Ophthalmol. 1988;106(5):640-5.

46. Peyman GA, Schulman JA, Sullivan B. Perfluorocarbon liquids in ophthalmology. Surv Ophthalmol. 1995;39(5):375-95. Review.

47. Alfaro DV, Liggett PF. Perfluorocarbon liquids in the management of traumatic retinal detachment: Advantage of PFCs in traumatic retinal detachment. Vitreoretinal Surg Technol. 1993;15:1-2.

48. Liu KR, Peyman GA, Chen MS, Chang KB. Use of high-density vitreous substitutes in the removal of posteriorly dislocated lenses or intraocular lenses. Ophthalmic Surg. 1991;22(9):503-7.

49. Rowson NJ, Bacon AS, Rosen PH. Perfluorocarbon heavy liquids in the management of posterior dislocation of the lens nucleus during phacoemulsification. Br J Ophthalmol. 1992;76(3):169-70.

50. Shapiro MJ, Resnick KI, Kim SH, Weinberg A. Management of the dislocated crystalline lens with a perfluorocarbon liquid. Am J Ophthalmol. 1991;112(4): 401-5.

51. Wallace RT, McNamara JA, Brown G, et al. The use of perfluorophenanthrene in the removal of intravitreal lens fragments. Am J Ophthalmol. 1993;116(2): 196-200.

52. Brod RD, Flynn HW Jr, Clarkson JG, Blankenship GW. Management options for retinal detachment in the presence of a posteriorly dislocated intraocular lens. Retina. 1990;10(1):50-6.

53. Lewis H, Blumenkranz MS, Chang S. Treatment of dislocated crystalline lens and retinal detachment with perfluorocarbon liquids. Retina. 1992;12(4): 299-304.

54. Chiang A, Garg SJ, Alshareef RA, et al. Removal of posterior segment retained lens material using the ozil phacoemulsification handpiece versus fragmatome during pars plana vitrectomy. Retina. 2012;32(10):2119-26.

55. Friedberg MA, Berler DK. Scleral fixation of posterior chamber intraocular lens implants combined with vitrectomy. Ophthalmic Surg. 1992 Jan;23(1): 17-21.

56. Lee SJ, Kim IG, Park JM. Management of posteriorly dislocated crystalline lens with perfluorocarbon liquid and fibrin glue-assisted scleral-fixated intraocular lens implantation. J Cataract Refract Surg. 2013;39(3):334-8.

57. Dalma-Weiszhausz J, Franco-Cardenas V, Dalma A. A modified technique for extracting a dislocated lens with perfluorocarbon liquids and viscoelastics. Ophthalmic Surg Lasers Imaging. 2010;41(5):572-4.

58. Lakhanpal V. Experimental and clinical observations on massive suprachoroidal hemorrhage. Trans Am Ophthalmol Soc. 1993;91:545-652.

59. Reynolds MG, Haimovici R, Flynn HW Jr, et al. Suprachoroidal hemorrhage. Clinical features and results of secondary surgical management. Ophthalmology. 1993;100(4):460-5.

60. Lakhanpal V, Schocket SS, Elman MJ, Nirankari VS. A new modified vitreoretinal surgical approach in the management of massive suprachoroidal hemorrhage. Ophthalmology. 1989;96(6):793-800.

61. Desai UR, Peyman GA, Chen CJ, et al. Use of perfluoroperhydrophenanthrene in the management of suprachoroidal hemorrhages. Ophthalmology. 1992;99(10):1542-7.

62. Glatt H, Machemer R. Experimental subretinal hemorrhage in rabbits. Am J Ophthalmol 1982;94:762-773.

63. Scupola A, Coscas G, Soubrane G, Balestrazzi E. Natural history of macular subretinal hemorrhage in age-related macular degeneration. Ophthalmologica. 1999;213(2):97-102.

64. Toth CA, Morse LS, Hjelmeland LM, Landers MB III: Fibrin directs early retinal damage after experimental subretinal hemorrhage. Arch Ophthalmol. 1991;109:723-729.

65. Hanscom TA, Diddie KR. Early surgical drainage of macular subretinal hemorrhage. Arch Ophthalmol. 1987;105(12):1722-3.

66. Johnson MW, Olsen KR, Hernandez E. Tissue plasminogen activator thrombolysis during surgical evacuation of experimental subretinal hemorrhage. Ophthalmology. 1992;99(4):515-21.

67. Vander JF. Tissue plasminogen activator irrigation to facilitate removal of subretinal hemorrhage during vitrectomy. Ophthalmic Surg. 1992;23(5):361-3.

68. Heriot WJ: Intravitreal gas and TPA: An outpatient procedure for submacular hemorrhage. American Academy of Ophthalmology Annual Vitreoretinal Update, 1996.

69. Haupert CL, McCuen BW, Jaffe GJ, et al. Pars plana vitrectomy, subretinal injection of tissue plasminogen activator, and fluid-gas exchange for displacement of thick submacular hemorrhage in age-related macular degeneration. Am J Ophthalmol. 2001;131:208-215.

70. Hillenkamp J, Surguch V, Framme C, et al. Management of submacular hemorrhage with intravitreal versus subretinal injection of recombinant tissue plasminogen activator. Graefes Arch Clin Exp Ophthalmol. 2010;248:5-11.

71. Olivier S, Chow DR, Packo KH, et al. Subretinal recombinant tissue plasminogen activator injection and pneumatic displacement of thick submacular hemorrhage in age-related macular degeneration. Ophthalmology. 2004;111:1201-1208.

72. Meyer CH, Scholl HP, Eter N, et al. Combined treatment of acute subretinal hemorrhages with intravitreal recombined tissue plasminogen activator, expansile gas and bevacizumab: A retrospective pilot study. Acta Ophthalmol. 2008;86:490-494.

73. MacLaren RE, Uppal GS, Balaggan KS, et al. Autologous transplantation of the retinal pigment epithelium and choroid in the treatment of neovascular age-related macular degeneration. Ophthalmology. 2007;114(3):561-70.

74. Han L, Ma Z, Wang C, et al. Autologous transplantation of simple retinal pigment epithelium sheet for massive submacular hemorrhage associated with pigment epithelium detachment. Invest Ophthalmol Vis Sci. 2013;54(7): 4956-63.

75. van Zeeburg EJ, van Meurs JC. Literature review of recombinant tissue plasminogen activator used for recent-onset submacular hemorrhage displacement in age-related macular degeneration. Ophthalmologica. 2013;229(1):1-14.

76. Mathis A. The use of perfluorodecalin in diabetic vitrectomy. J Vitreo Retina. 1992;1:28-29.

77. Corcostegui B. Use of perfluorocarbon liquid in vitrectomy for diabetic rhegmatogenous retinal detachment. J Vitreo Retina. 1992;1:30-34.

78. Le Mer Y. Use of liquid perfluorocarbons in vitrectomy for difficult cases of proliferative diabetic retinopathy. J Fr Ophtalmol. 1995;18(5):366-72.

79. Chiquet C, Thuret G. Perfluorocarbon liquids and vitreoretinal surgery in 2011. J Fr Ophtalmol. 2011;34(9):663-77.

80. Barzideh N, Johnson TM. Subfoveal fluid resolves slowly after pars plana vitrectomy for tractional retinal detachment secondary to proliferative diabetic retinopathy. Retina. 2007;27(6):740-3.

81. Kertes PJ, Wafapoor H, Peyman GA, et al. The management of giant retinal tears using perfluoroperhydrophenanthrene. A multicenter case series. Vitreon Collaborative Study Group. Ophthalmology. 1997;104(7):1159-65.

82. Scott IU, Flynn HW Jr, Murray TJ, et al. Outcomes of surgery for retinal detachment associated with proliferative vitreoretinopathy using perfluoro-n-octane: A multicenter study. Perfluoron Study Group. Am J Ophthalmol. 2003;136(3):454-63.

83. Scott IU, Murray TJ, Flynn HW Jr, et al. Outcomes and complications associated with giant retinal tear management using perfluoro-n-octane. Perfluoron Study Group. Ophthalmology. 2002;109(10):1828-33.

84. Yoshida A, Ho PC, Schepens CL, et al. Severe proliferative vitreoretinopathy and retinal detachment. II. Surgical results with scleral buckling. Ophthalmology. 1984;91(12):1538-43.

85. Sternberg P Jr, Machemer R. Results of conventional vitreous surgery for proliferative vitreoretinopathy. Am J Ophthalmol. 1985;100(1):141-6.

86. Quinlan E, Haller JA. Perfluorocarbon liquids in vitreoretinal surgery. Retinal Physician. Issue: November 2006.

87. Scott IU, Murray TG, Flynn HW Jr, et al. Outcomes and complications associated with perfluoro-n-octane and perfluoroperhydrophenanthrene in complex retinal detachment repair. Ophthalmology. 2000;107(5):860-5.

88. Loewenstein A, Humayun MS, de Juan E Jr, et al. Perfluoroperhydrophenanthrene versus perfluoro-n-octane in vitreoretinal surgery. Ophthalmology. 2000;107(6):1078-82.

89. Crafoord S, Larsson J, Hansson LJ, et al. The use of perfluorocarbon liquids in vitreoretinal surgery. Acta Ophthalmol Scand. 1995;73(5):442-5.

90. Mathis A, Pagot V, Gazagne C, Malecaze F. Giant Retinal Tears. Surgical Techniques and Results Using Perfluorodecalin and Silicone Oil Tamponade. Retina. 1992;12(3 Suppl):S7-10.

91. Mandai M, Takanashi T, Ogura Y, Honda Y. Proliferative vitreoretinopathy shows predilection for the inferior fundus. Graefes Arch Clin Exp Ophthalmol. 1990;228(4):335-7.

92. Bottoni F, Sborgia M, Arpa P, et al. Perfluorocarbon liquids as postoperative short-term vitreous substitutes in complicated retinal detachment. Graefes Arch Clin Exp Ophthalmol. 1993;231(11):619-28.

93. Rofail M, Lee LR. Perfluoro-n-octane as a postoperative vitreoretinal tamponade in the management of giant retinal tears. Retina. 2005;25(7): 897-901.

94. Sirimaharaj M, Balachandran C, Chan WC, et al. Vitrectomy with short term postoperative tamponade using perfluorocarbon liquid for giant retinal tears. Br J Ophthalmol. 2005;89(9):1176-9.

95. Drury B, Bourke RD. Short-term intraocular tamponade with perfluorocarbon heavy liquid. Br J Ophthalmol. 2011;95(5):694-8.

96. Chang S, Zimmerman NJ, Iwamoto T, et al. Experimental vitreous replacement with perfluorotributylamine. Am J Ophthalmol. 1987;103(1): 29-37.

97. Chang S, Sparrow JR, Iwamoto T, et al. Experimental studies of tolerance to intravitreal perfluoro-n-octane liquid. Retina. 1991;11(4):367-74.

98. Nakamura K, Refojo MF, Crabtree DV. Factors contributing to the emulsification of intraocular silicone and fluorosilicone oils. Invest Ophthalmol Vis Sci. 1990;31(4):647-56.

99. Sigler EJ, Randolph JC, Calzada JI, Charles S. Pars plana vitrectomy with medium-term postoperative perfluoro-n-octane for recurrent inferior retinal detachment complicated by advanced proliferative vitreoretinopathy. Retina. 2013;33(4):791-7.

100. Grafstein D. Detection, estimation and removal of impurities in fluorocarbon liquids. Anal Chem. 1954;26:523.

101. Keese CR, Giaever I. Cell growth on liquid interfaces: Role of surface active compounds. Proc Natl Acad Sci USA. 1983;80:5622.

102. Maroudas NG. Chemical and mechanical requirements for fibroblast adhesion. Nature. 1973;244(5415):353-4.

103. Giaver I, Keese CR. Behaviour of cells at fluid interfaces. Proc Natl Acad Sci USA. 1983;80:219.

104. Sparrow JR, Ortiz R, MacLeish PR, Chang S. Fibroblast behavior at aqueous interfaces with perfluorocarbon, silicone, and fluorosilicone liquids. Invest Ophthalmol Vis Sci. 1990;31(4):638-46.

105. Velikay M, Wedrich A, Stolba U, et al. Experimental long-term vitreous replacement with purified and nonpurified perfluorodecalin. Am J Ophthalmol. 1993;116(5):565-70.

106. Velikay M, Stolba U, Wedrich A, et al. The effect of chemical stability and purification of perfluorocarbon liquids in experimental extended-term vitreous substitution. Graefes Arch Clin Exp Ophthalmol. 1995;233(1):26-30.

107. Eckardt C, Nicolai U, Winter M, Knop E. Experimental intraocular tolerance to liquid perfluorooctane and perfluoropolyether. Retina. 1991;11(4):375-84.

108. Gonvers M, Hornung JP, de Courten C. The effect of liquid silicone on the rabbit retina. Histologic and ultrastructural study. Arch Ophthalmol. 1986;104(7):1057-62.

109. Orzalesi N, Migliavacca L, Bottoni F, Miglior S. Experimental short-term tolerance to perfluorodecalin in the rabbit eye: a histopathological study. Curr Eye Res. 1998;17(8):828-35.

110. Devin F, Jourdan T, Saracco JB, Lucciani A. Experimental tolerance to perfluorodecalin used in prolonged intraocular tamponade. Ophthalmologica. 1995;209(6):306-14.

111. Millsap CM, Peyman GA, Mehta NJ, el al. Perfluoroperhydrophenanthrene (Vitreon) in the management of giant retinal tears: results of a collaborative study. Ophthalmic Surg. 1993;24(11):759-63.

112. Stolba U, Krepler K, Pflug R, et al. Experimental vitreous and aqueous replacement with perfluorophenanthrene. Clinical, histologic, and electrophysiologic results. Retina. 1997;17(2):146-53.

113. Batman C, Cekic O. Effects of the long-term use of perfluoroperhydrophenanthrene on the retina. Ophthalmic Surg Lasers. 1998;29(2):144-6.

114. Stolba U, Krepler K, Velikay-Parel M, Binder S. The effect of specific gravity of perfluorocarbon liquid on the retina after experimental vitreous substitution. Graefes Arch Clin Exp Ophthalmol. 2004;242(11):931-6.

115. Moreira H, de Queiroz JM Jr, Liggett PE, McDonnell PJ. Corneal toxicity study of two perfluorocarbon liquids in rabbit eyes. Cornea. 1992;11(5):376-9.

116. Meyer MA, Zak RD. The Effect of Perfluorophenanthrene (PFP, Vitreon®) Liquid on the Duration of Intraocular Perfluorocarbon Gas (Abstract). Invest Ophthalmol Vis Sci. 1994;35(suppl):1617.

117. Peyman GA, Conway MD, Soike KF, Clark LC Jr. Long-term vitreous replacement in primates with intravitreal Vitreon or Vitreon plus silicone. Ophthalmic Surg. 1991;22(11):657-64.

118. Sparrow JR, Jayakumar A, Berrocal M, et al. Experimental studies of the combined use of vitreous substitutes of high and low specific gravity. Retina. 1992;12(2):134-40.

119. Ciardella AP, Langton K, Chang S. Intraocular dispersion of perfluorocarbon liquids in silicone oil. Am J Ophthalmol. 2003;136(2):365-7.

120. Mathis A, Pagot V, Idder A, Malecaze F. Use of perfluorodecalin during vitrectomy in diabetics. J Fr Ophtalmol. 1993;16(11):584-90. Article in French.

121. Garcia-Valenzuela E, Ito Y, Abrams GW. Risk factors for retention of subretinal perfluorocarbon liquid in vitreoretinal surgery. Retina. 2004;24(5): 746-52.

122. Blinder KJ, Peyman GA, Paris CL, et al. Vitreon, a new perfluorocarbon. Br J Ophthalmol. 1991;75(4):240-4.

123. Elsing SH, Fekrat S, Green WR, et al. Clinicopathologic findings in eyes with retained perfluoro-n-octane liquid. Ophthalmology. 2001;108(1):45-8.

124. Alster Y, Ben-Nun Y, Loewenstein A, Lazar M. Pupillary block glaucoma due to residual perfluoro-decalin. Ophthalmic Surg Lasers. 1996;27(5):395-6.

125. Green K, Cheeks L, Friedman JM. Perfluorocarbon liquid effects on corneal endothelial permeability. Lens Eye Toxic Res. 1992;9(1):1-8.

126. Stolba U, Krepler K, Velikay M, Binder S. Anterior segment changes in rabbits after experimental aqueous replacement with various amounts of different perfluorocarbon liquids. Graefes Arch Clin Exp Ophthalmol. 1999;237(6): 501-7.

127. Wilbanks GA, Apel AJ, Jolly SS, et al. Perfluorodecalin corneal toxicity: five case reports. Cornea. 1996;15(3):329-34.

128. Cauchi P, Azuara-Blanco A, McKenzie J. Corneal toxicity and inflammation secondary to retained perfluorodecalin. Am J Ophthalmol. 2005;140(2):322-3.

129. Ramaesh K, Bhagat S, Wharton SB, Singh J. Corneal epithelial toxic effects and inflammatory response to perfluorocarbon liquid. Arch Ophthalmol. 1999 Oct;117(10):1411-3.

130. Bourke RD, Simpson RN, Cooling RJ, Sparrow JR. The stability of perfluoro-n-octane during vitreoretinal procedures. Arch Ophthalmol. 1996;114(5): 537-44.

131. Fujii GY, De Juan E Jr, Humayun MS, et al. A new 25-gauge instrument system for transconjunctival sutureless vitrectomy surgery. Ophthalmology. 2002;109(10):1807-12; discussion 1813. Erratum in: Ophthalmology. 2003;110(1):9.

132. Eckardt C. Transconjunctival sutureless 23-gauge vitrectomy. Retina. 2005;25(2):208-11.

133. Lakhanpal RR, Humayun MS, de Juan E Jr, et al. Outcomes of 140 consecutive cases of 25-gauge transconjunctival surgery for posterior segment disease. Ophthalmology. 2005;112(5):817-24.

134. Hubschman JP, Gupta A, Bourla DH, et al. 20-, 23-, and 25-gauge vitreous cutters: performance and characteristics evaluation. Retina. 2008;28(2): 249-57.

135. Kunikata H, Abe T, Nishida K. Successful outcomes of 25- and 23-gauge vitrectomies for giant retinal tear detachments. Ophthalmic Surg Lasers Imaging. 2011;42(6):487-92.

136. Garg SJ, Theventhiran AB. Retained subretinal perfluorocarbon liquid in microincision 23-gauge versus traditional 20-gauge vitrectomy for retinal detachment repair. Retina. 2012;32(10):2127-32.

137. Roth DB, Sears JE, Lewis H. Removal of retained subfoveal perfluoro-n-octane liquid. Am J Ophthalmol. 2004;138(2):287-9.

138. García-Arumí J, Castillo P, López M, et al. Removal of retained subretinal perfluorocarbon liquid. Br J Ophthalmol. 2008;92(12):1693-4.

139. Berglin L, Ren J, Algvere PV. Retinal detachment and degeneration in response to subretinal perfluorodecalin in rabbit eyes. Graefes Arch Clin Exp Ophthalmol. 1993;231(4):233-7.

140. Lee GA, Finnegan SJ, Bourke RD. Subretinal perfluorodecalin toxicity. Aust N Z J Ophthalmol. 1998;26(1):57-60.

141. Inoue M, Iriyama A, Kadonosono K, et al. Effects of perfluorocarbon liquids and silicone oil on human retinal pigment epithelial cells and retinal ganglion cells. Retina. 2009;29(5):677-81.

142. Lesnoni G, Rossi T, Gelso A. Subfoveal liquid perfluorocarbon. Retina. 2004;24(1):172-6.

143. Tewari A, Eliott D, Singh CN, et al. Changes in retinal sensitivity from retained subretinal perfluorocarbon liquid. Retina. 2009;29(2):248-50.

144. Nishimura A, Kita K, Segawa Y, Shirao Y. Perfluorocarbon liquid assists in stripping the ILM to treat detached retina caused by macular hole. Ophthalmic Surg Lasers. 2002;33(1):77-8.

145. Brazitikos PD, Androudi S, Dimitrakos SA, Stangos NT. Removal of the internal limiting membrane under perfluorocarbon liquid to treat macular-hole-associated retinal detachment. Am J Ophthalmol. 2003;135(6):894-6.

146. Rizzo S, Belting C, Genovesi-Ebert F, Hagedorn N. Colored perfluorocarbon liquids as novel intraoperative tools. Graefes Arch Clin Exp Ophthalmol. 2012;250(5):653-9.

147. Shentu X, Tang X, Ye P, Yao K. Combined microwave energy and fixative agent for cataract induction in pig eyes. J Cataract Refract Surg. 2009;35(7): 1150-1155.

148. Yang P, Chen L, Zwart R, Kijlstra A. Immune cells in the porcine retina: distribution, characterization and morphological features. Invest Ophthalmol Vis Sci. 2002;43(5):1488-92.

149. Ruiz-Ederra J, García M, Hernández M, et al. The pig eye as a novel model of glaucoma. Exp Eye Res. 2005;81(5):561-569.

150. Gesslein B, Håkansson G, Gustafsson L, et al. Tumor necrosis factor and its receptors in the neuroretina and retinal vasculature after ischemia-reperfusion injury in the pig retina. Mol Vis. 2010;16:2317-2327.

151. Lopez-Guajardo L, Benitez-Herreros J, Silva-Mato A. Experimental model to evaluate mechanical closure resistance of sutureless vitrectomy sclerotomies using pig eyes. Invest Ophthalmol Vis Sci. 2011;52(7):4080-4084.

152. Rootman J. Vascular system of the optic nerve head and retina in the pig. Br J Ophthalmol. 1971;55(12):808-19.

153. Azzolini C, Docchio F, Brancato R, Trabucchi G. Interactions between light and vitreous fluid substitutes. Arch Ophthalmol. 1992;110(10):1468-71.

154. Sparrow JR, Matthews GP, Iwamoto T, et al. Retinal tolerance to intravitreal perfluoroethylcyclohexane liquid in the rabbit. Retina. 1993;13(1):56-62.

155. Prince JH, McConnell DG: Retina and optic nerve. In The rabbit in eye research, Prince JH (Ed.). Springfield, IL: Charles C. Thomas, 1964, pp. 385-449.

156. Middleton S. Porcine ophthalmology. Vet Clin North Am Food Anim Pract. 2010;26(3):557-72.

157. Momirov D, van Lith GH, Zivojnović R. Electroretinogram and electrooculogram of eyes with intravitreously injected silicone oil. Ophthalmologica. 1983;186(4):183-8.

158. Foerster MH, Esser J, Laqua H. Silicone oil and its influence on electrophysiologic findings. Am J Ophthalmol. 1985;99(2):201-6.

159. Doslak MJ. A theoretical study of the effect of silicone oil on the electroretinogram. Invest Ophthalmol Vis Sci. 1988 Dec;29(12):1881-4.

160. Schechner R, Gdal-on M, Cohen D, et al. Recovery of the electroretinogram in rabbits after argon laser photocoagulation. Invest Ophthalmol Vis Sci. 1987;28(9):1605-13.

161. Bonnet D, Garcia M, Vecino E, et al. Brain-derived neurotrophic factor signalling in adult pig retinal ganglion cell neurite regeneration in vitro. Brain Res. 2004;1007(1-2):142-51.

162. Balaiya S, Brar VS, Murthy RK, Chalam KV. Comparative in vitro safety analysis of dyes for chromovitrectomy: indocyanine green, brilliant blue green, bromophenol blue, and infracyanine green. Retina. 2011;31(6):1128-36.

163. Iriyama A, Kadonosono K, Tamaki Y, Yanagi Y. Effect of Brilliant Blue G on the retinal ganglion cells of rats. Retina. 2012;32(3):613-6.

164. Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol. 1993;170(1):1-8.

165. Pulkki AS, Voipio-Pulkki LM. Significance of myocytes with positive DNA in situ nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy. Circulation. 2000;101(25):E239.

166. Kanoh M, Takemura G, Misao J, et al. Comment on: Significance of myocytes with positive DNA in situ nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy: not apoptosis but DNA repair. Circulation. 1999;99(21): 2757-64.

167. Hiscott P, Magee RM, Colthurst M, et al. Clinicopathological correlation of epiretinal membranes and posterior lens opacification following perfluorohexyloctane tamponade. Br J Ophthalmol. 2001;85(2):179-83.

168. Roider J, Hoerauf H, Kobuch K, Gabel VP. Clinical findings on the use of long-term heavy tamponades (semifluorinated alkanes and their oligomers) in complicated retinal detachment surgery. Graefes Arch Clin Exp Ophthalmol. 2002;240(12):965-71. Epub 2002 Nov 6.

169. Mertens S, Bednarz J, Engelmann K. Evidence of toxic side effects of perfluorohexyloctane after vitreoretinal surgery as well as in previously established in vitro models with ocular cell types. Graefes Arch Clin Exp Ophthalmol. 2002;240(12):989-95.

170. Hoerauf H, Laqua H. Severe emulsification after combined use of a partially fluorinated alkane and silicone oil. Graefes Arch Clin Exp Ophthalmol. 2002;240(2):131-6.

171. Majid MA, Hussin HM, Biswas S, et al. Emulsification of Densiron-68 used in inferior retinal detachment surgery. Eye (Lond). 2008;22(1):152-7.

172. Rizzo S. The potential surgical benefits of colored perfluorocarbon. Retina Today. Issue: March 2011.