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Article

Evaluation of Cytotoxicity of Perfluorocarbons for Intraocular Use by Cytotoxicity Test In Vitro in Cell Lines and Human Donor Retina Ex Vivo

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Purpose: To validate the cytotoxicity test of perfluorocarbon liquids (PFCLs) for intraocular use according to the ISO 10993-5 standard.

Methods: BALB/3T3, ARPE-19 cell lines, and 3-mm human retina ex vivo samples were cultured in 96-well plates. Contact areas of 22%, 59%, and 83% and 2.5-, 12-, and 24-hour contact times were tested in cell lines. Cell viability was quantified by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in ARPE-19 and neutral red uptake (NRU) viability assay for BALB/3T3. Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in ARPE-19 cells. 1-H perfluorooctane (1H PFO) and purified perfluorooctane (PFO) were used as cytotoxic and not cytotoxic controls, respectively. Cell viability was assessed by MTT assay in retina ex vivo samples.

Results: Qualitative evaluation showed that cytotoxic control induced apoptosis, severe reactivity zones, and cytotoxicity according to ISO 10993-5 in all tested conditions. Quantitative evaluation of 1H PFO showed no cytotoxicity according to ISO 10993-5 on 22% areas, whereas cytotoxicity was detected on 59%, and 83% contact areas. The PFO was confirmed not to be cytotoxic in all tested conditions. Quantitative evaluation in retina ex vivo samples confirmed no cytotoxicity with PFO and cytotoxicity with 1H PFO.

Conclusions: The direct contact cytotoxicity test according to ISO 10993-5 is a suitable method to detect the cytotoxicity of PFCLs and was validated using quantitative and qualitative approaches in ARPE-19 and BALB/3T3 cells covering 59% of the cell surface areas for 24 hours.

Translational Relevance: Direct contact cytotoxicity test using specific conditions was validated, whereas different test conditions could not be validated.

Introduction

The perfluorocarbon liquids (PFCLs) are synthetic liquid fluorinated carbon-containing compounds, first investigated and used as vitreous tamponade in the 1980s.[1,2] Physically PFCLs are characterized by specific gravity greater than water (ranging from 1.7–2.03 g/cm^3);[3] moderate interfacial tension (approximately 50 mN/m against water); and low viscosity, transparency, and immiscibility with water.[4] Due to the aforementioned properties, PFCLs unfold and keep the detached retina flat, concurrently with the anterior displacement of the subretinal fluid, allowing to perform other surgical maneuvers, such as membrane removal or photocoagulation.[5] Therefore, they are valid intraoperative tools for surgical treatment of complex retinal detachments (RDs), especially in the presence of severe proliferative vitreoretinopathy (PVR), giant retinal tears, funnel-

shaped RDs, and proliferative diabetic retinopathy.[5,6] Moreover, PFCLs are used off-label for primary RDs, dropped lenses with or without RD, removal of foreign bodies, drainage of suprachoroidal hemorrhage, and management of submacular hemorrhage.[6,7] Despite the potential advantages of such high specific gravity, these compounds are not used as long-term tamponade because their persistence in the vitreous cavity has been associated with irreversible retinal toxicity, emulsification, and inflammation.[8,9] Therefore, careful removal of PFCL not only is crucial, but also challenging as we already reported describing the induced foreign body response consequent to intraoperative use of PFCL and the accumulation of residual droplets of PFCL 10 minutes after the air–fluid exchange.[10,11] Currently, the most largely used PFCLs are perfluorodecalin (PFD) and perfluoro-n-octane (PFO). In particular, PFO, as a saturated fluorinated compound, is considered chemically and biologically inert and its safe profile has been variously documented.[7,12,13] The safety of PFO and PFD, medical devices intended for intraocular use is an essential requirement according to European[14–16] and US regulations.[15–17] Nevertheless, recently Pastor et al.[18] reported 117 cases of severe retinal acute toxicity, mostly characterized by retinal necrosis and vascular occlusion, after intraoperative use of PFO Ala-Octa (Alamedics, Dornstadt, Germany). This PFO was certified as safe by a German company based on an extract cytotoxicity test.[15] Performing a counter-analysis of some unused toxic lots of PFO Ala-Octa, the Spanish Instituto de Oftalmobiología Aplicada (IOBA) determined their toxicity, identifying benzene derivatives, perfluorooctanoic acid and dodecafluoro-1-heptanol as suspected causative agents.[18] IOBA used human retinal pigment epithelial cells (ARPE-19 cells) in direct contact methods.[15,18] Showing their results, Pastor et al.[18] and Srivastava et al.[19] declaimed the failure and inadequacy of the ISO 10993-5 criteria and proposed a different protocol for the evaluation of cytotoxicity.

As clearly stated in the introduction of "Biological evaluation of medical devices – Tests for in vitro cytotoxicity,"[15] considering the great number of medical devices and the consequent widespread use and applicability of the in vitro cytotoxicity tests, the aim of the ISO is to provide a scheme to perform the test correctly, rather than oblige the selection of tests to be performed for each medical device, such as PFCL. Obviously, test conditions must be critically reviewed and validated. We herein validated the cytotoxicity test of PFCLs in vitro according to ISO 10993-5 (2009).[15]

Material and Methods

Validation Study Flowchart

The validation study was conducted according to the flowchart shown in Supplementary Figure S1.

Cell Cultures

Two cell lines were included in the validation study: the ISO 10993-5 reference cell line, BALB/3T3, and ARPE-19 cell line, which simulate the cell type that comes in direct contact with PFCL during retina surgery. The murine fibroblast cells BALB/3T3 clone A31 (ATCC CCL163) and the human retinal pigment epithelial cell line ARPE-19 (ATCC CRL-2302) were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). After thawing, BALB/3T3 and ARPE-19 cells were grown in Dulbecco's modified eagle's medium (DMEM) with high glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate (Sigma-Aldrich Corp., St. Louis, MO) and DMEM/Nutrient Mixture F-12 medium with L-glutamine without (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco, Monza, Italy), respectively, each supplemented with 10% of iron-fortified bovine calf serum (Sigma-Aldrich Corp.) and 1% penicillin-streptomycin (Sigma-Aldrich Corp.), as a monolayer in 75 cm^2 culture flasks at 37°C ± 1°C, 90% ± 10% humidity, and 5.0% ± 1.0% CO_2/air. The medium was changed every 48 hours. At 80% confluence, cells were removed from the flask by trypsinization. Single cell suspension was counted manually using trypan blue staining and a hemocytometer. The counting was repeated before each cell passage or seeding in 96-well plates. After thawing, cells were passaged two times before using them in the test.

Human Donor Retina Ex Vivo

Human donor eye globes for research use were recovered and transported by Fondazione Banca degli Occhi del Veneto (FBOV, Zelarino, VE, Italy). The eye globes were stored in DMEM/F-12 medium at 4°C for 24 hours, corresponding to the time of death to retina extraction. Retina was extracted without retinal pigment epithelium - choroid. A 3-mm diameter biopsy punch (Kai Medical, Solingen, Germany) was used for retina sampling.

Direct Contact Cytotoxicity Test Conditions

BALB/3T3 and ARPE-19 cell suspension containing 2.0 to 3.0 $\times$ 10^5 cells/mL was seeded into 96-well microtiter plates and placed in an incubator (Memmert, Schwabach, Germany) for 24 hours. Each plate was examined under a phase contrast microscope (Leica DM IL LED, Wetzlar, Germany) for even cell growth and 70% to 80% confluence. The cells were washed one time with 150 µL of Dulbecco's phosphate-buffered saline before application of test samples.

Vehicle media for BALB/3T3 cells and ARPE-19 cells were the media respectively adopted for cell culture growth. Dulbecco's phosphate-buffered saline with $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich Corp.) was used as washing medium.

Cytotoxic And Not Cytotoxic Controls

PFCLs are heavy liquids that may induce cell mortality by excessive pressure exerted on the cell layer because of the sample weight. To control this possible effect of PFCLs during direct contact test, the PFO and 1H PFO were tested as not cytotoxic and cytotoxic controls, respectively, under different time and dose conditions. Ultrapure PFO, purity 99.8%, was purchased from AL.CHI.MI.A Srl (Ponte san Nicolo, Italy), and was used as not cytotoxic control. 1H PFO, with purity 98.8%, was purchased from Fluorochem (Hadfield, UK), and used as cytotoxic control. The density of PFO and 1HPFO was 1.8 g/cm^3.

Contact Time and Contact Area Validation

According to the "direct contact method" of the ISO 10993-5 (2009),[15] the application time corresponds to 24 hours of direct contact between sample and the cell layer. We investigated PFCL application time using the cytotoxic control (1H PFO) and the not cytotoxic control (PFO) applied to the cell layer of both cell lines for 2.5, 12, and 24 hours.

According to the ISO 10993-5 (2009),[15] the contact area between the sample and cell layer should correspond to one-tenth of the cell layer surface. We investigated the PFCL contact area by application of 15, 50, and 80 µL 1H PFO (cytotoxic control) and PFO (not cytotoxic control). We previously established that the tested volumes of 15, 50, and 80 µL corresponded, respectively, to 22%, 59%, and 83% of contact area between PFO sample and cell layer in 96-well plates.

Application and Removal of PFCL Samples

PFCLs are uncolored, transparent, volatile, heavy liquids that are totally immiscible with aqueous solutions. We previously established a method for PFCL direct deposition on the cell layer to guarantee the direct contact between the PFCL sample and cell layer for the whole duration of the test and to avoid sample evaporation or loss of PFCL contact with the cell layer. In particular, we used cells at 70% to 80% of confluence, cultured in 96-well plates with 300 µL of the medium. PFCL samples were deposited with the tip immersed in the medium at 2/3 depth of the well to form a single bubble that remained in constant contact with the cell layer because of the force of gravity mimicking the clinical use (Supplementary Movie S1). At the end of the incubation time, the PFCL bubble was removed together with the medium.

3-(4,5-Dimethylthiazole-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and Neutral Red Uptake (NRU) Vitality Assays

The MTT cytotoxicity test was used with the ARPE-19 cell line. The assay uses yellow water-soluble MTT, which is metabolically reduced in viable cells to a blue-violet insoluble formazan. The number of viable cells correlates with the color intensity determined by photometric measurements after dissolving the formazan in alcohol. In the absence of ISO 10993-5 indications for ARPE-19 cells, the TOX-1 in vitro toxicology assay kit, MTT-based (Sigma-Aldrich Corp.) was used and the equivalence between MTT and NRU assays in terms of cell mortality response was studied for the ARPE-19 cell line, by testing 15, 50, and 80 µL 1H PFO (cytotoxic control) and PFO (not cytotoxic control) 24 hours after application.

The NRU cytotoxicity test was used with BALB/3T3 cells as suggested by ISO 10993-5 (2009).[15] Neutral red is a eurhodin dye that stains lysosomes in viable cells. Only viable cells incorporate the dye into their lysosomes. Consequently, after washing, fixing, and dye extraction, viable cells release the incorporated dye and the amount of released dye determines the total number of viable cells. The test was performed according to Annex 1 of the ISO 10993-5 (2009)[15] and using the TOX4 In vitro toxicology assay kit, Neutral Red-based (Sigma-Aldrich Corp.).

Table 1. Grading System for Evaluation of the Reactivity Zone Before and After NRU and MTT Viability Assays[15]

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 1.0 cm
4	Severe	Zone extending farther than 1.0 cm beyond specimen

Morphologic Evaluation of the Cells by Light Microscopy

The cells were examined microscopically (Leica DM IL LED) by two independent operators, before sample removal and after the phase of dye extraction and cell fixing in MTT or NRU assay. The changes in general morphology, vacuolization, detachment, cell lysis, and membrane integrity were assessed. The reactivity zone under and around the sample was graded according to Table 1. Grade 4 was not applicable in our testing conditions as the well diameter in 96 wells setting was inferior to 1 cm. The achievement of a numerical grade greater than 2, based on Table 1, was considered a cytotoxic effect.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

TUNEL assay was performed using In situ Cell Death Detection Kit, AP (Roche, Mannheim, Germany) according to the manufacturer's instructions to verify the presence of apoptotic cells after application of 12.5 % 1H PFO in ultrapure PFO (cytotoxic control, $n = 3$), ultrapure 99.8% PFO (not cytotoxic control, $n = 3$), and the vehicle medium ($n = 3$). The cells were examined microscopically (Leica DM IL LED) under and around the sample by two independent operators. Each condition was evaluated with ×400 magnification under and out of the sample according to the grading system reported in Table 2.

Table 2. Grading System for the Evaluation of the Reactivity Zone After TUNEL Assay

Grade	Reactivity	Description of Reactivity Zone
0	None	Absence of apoptotic cells
1	Slight	Presence of sporadic apoptotic cells
2	Moderate	Widespread presence apoptotic cells
3	Severe	Widespread presence of apoptotic cells with altered morphology and/ or acellular zones

Cell Viability in Human Retina Ex Vivo and ARPE-19 Cell Line Models

The tissue samples were placed in 96-wells dishes and 50 µL of ultrapure 99.8% PFO + 250 µL DMEM/F-12 medium, 50 µL of 12.5% 1H PFO + 250 µL DMEM/F-12 medium and 300 µL of DMEM/F-12 medium were added to the wells corresponding to the not cytotoxic, cytotoxic and vehicle controls, respectively. After 24 hours of incubation at 37°C, the samples were washed with DPBS and the cell viability was determined using the MTT based TOX-1 in vitro toxicology assay kit, (Sigma-Aldrich Corp.). The optical density at $\lambda = 570$ nm was measured by Absorbance Microplate Reader ELx80 (Biotek Instruments, Winooski, VT). The percentage of cell viability in ARPE-19 cell culture and donor retina samples was compared. The coefficient of variation (%CV) was calculated for each experimental condition (vehicle, cytotoxic control, not cytotoxic control) and considered as "good" for %CV ranging from 10 to 20, and as "acceptable" for %CV ranging from 20 to 30.

Data Analysis and Statistics

At least six values were acquired at each 96-well microplate for all samples (vehicle, cytotoxic control, and not cytotoxic control). Three 96-wells plates were used in each experiment, and each experiment was repeated in three different days. Mean percent of cell mortality and standard error of the mean (SEM) were calculated for each sample and experimental condition. The differences between groups (cell type, contact area, contact time, MTT vs. NRU, human retina ex vivo vs. ARPE-19 cells) in percent mortality was determined by Student's *t*-test or by the Mann-

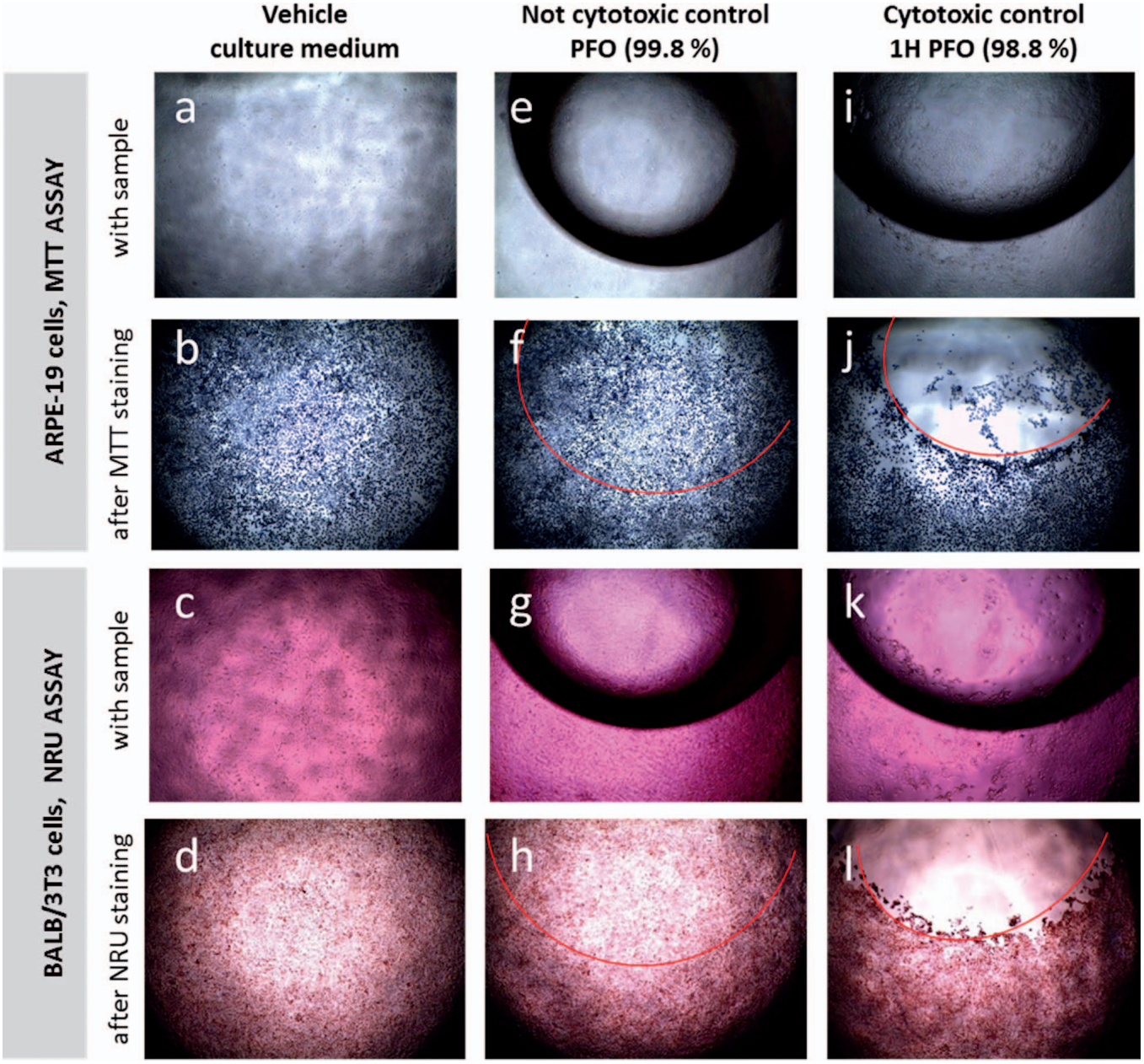


Figure 1. Qualitative cell viability evaluation after application of 50 μL vehicle, cytotoxic, and not cytotoxic controls for 24 hours of contact time. Pictures from light microscopy for vehicle (a–d); not cytotoxic control, PFO (e–h); and cytotoxic control, 1H PFO (i–l), in ARPE-19 and BALB/3T3 cell lines. The images were acquired in the presence of the sample before staining with MTT (a, e, i) or NRU (c, g, k) dyes and after the removal of the PFO or 1H PFO samples and the staining with MTT (b, f, g) or NRU dyes (d, h, l). The borders of the sample contact area are indicated with *red lines*.

Whitney *U* test in case of normal and not normal distribution of the sample groups respectively.

Results

Qualitative viability evaluation showed that in the presence of the cytotoxic control (1H PFO) ARPE-19 and BALB/3T3 cell layers were strongly altered, showing no cells or cells with irregular morphology under and close to 1H PFO sample contact area (Fig. 1). The cell morphology and density appeared mostly unchanged beyond the contact area with 1H PFO sample. Cell staining with vital dyes (MTT and NRU) confirmed the absence of viable cells under and close to the contact area (Figs. 1i–l) and the presence of a

Table 3. Grading Score ± SEM Obtained in Qualitative Evaluation of BALB/3T3 and ARPE-19 Cells During Viability Assay

Cell Line	Contact Area, % of Cell Layer	Contact Time, Hours	Not Cytotoxic Control PFO, 99.8%	Cytotoxic Control 1H PFO, 98.8%
BALB/3T3	22	2.5	0.1 ± 0.0	2.7 ± 0.1
		12	0.1 ± 0.0	2.4 ± 0.1
		24	0.1 ± 0.0	2.5 ± 0.1
	59	2.5	0.2 ± 0.1	2.7 ± 0.1
		12	0.9 ± 0.1	2.8 ± 0.1
		24	0.9 ± 0.1	3.0 ± 0.0
	83	2.5	0.3 ± 0.1	2.8 ± 0.1
		12	1.2 ± 0.1	2.9 ± 0.0
		24	1.2 ± 0.1	3.0 ± 0.0
ARPE-19	22	2.5	0.0 ± 0.0	1.7 ± 0.1
		12	0.1 ± 0.0	2.2 ± 0.1
		24	0.0 ± 0.0	2.3 ± 0.1
	59	2.5	0.1 ± 0.0	2.3 ± 0.1
		12	0.2 ± 0.1	2.8 ± 0.1
		24	0.0 ± 0.0	2.7 ± 0.1
	83	2.5	0.1 ± 0.0	2.4 ± 0.1
		12	0.3 ± 0.1	2.9 ± 0.1
		24	0.3 ± 0.1	2.9 ± 0.0

viable cell layer with 100% confluence in the rest of the well. The mean morphologic grading score ranged from 2.2 to 3.0 corresponding to the presence of severe reactivity zones in all tested conditions (Table 3).

Both cell lines incubated with the not cytotoxic control (PFO) on 22% contact area showed normal morphology and 100% confluence under or around the sample at all tested time intervals. In 51% and 89% contact areas for 12- and 24-hour contact times, the BALB/3T3 cells immediately under the sample showed a flatter shape, and the morphologic grading ranged from 1.0 to 1.4 (Table 3). The whole cell layer, including the areas under the not cytotoxic control sample, were viable in both cell lines as confirmed after cell staining with MTT or NRU vital dyes (Figs. 1e–h).

ARPE-19 and BALB/3T3 cell lines showed normal morphology and approximately 100% confluence after incubation with the vehicle medium for 24 hours (Figs. 1a–d) and the mean morphologic grading was 0.0 ± 0.0 in all the tested conditions (Table 3). According to ISO 10993-5 (2009)[15] qualitative evaluation, 1H PFO was cytotoxic in all tested conditions, whereas the vehicle medium and the PFO were not cytotoxic in all tested conditions.

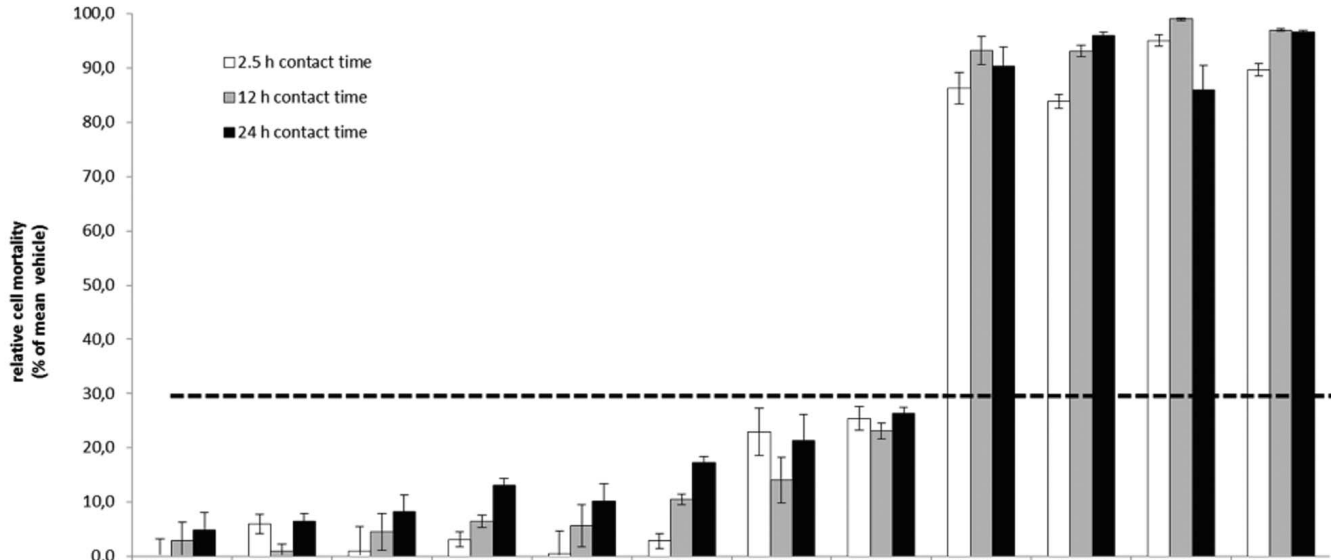
Figure 2 summarizes the results of quantitative viability evaluation. BALB/3T3 cell mortality ranged from 14% to 23% for all tested time intervals, after application of the cytotoxic control (1H PFO) on the 22% cell area. The cytotoxic control applied on the 22% area of the ARPE-19 cell layer induced 23% to 26% cell mortality. Under these testing conditions, 1H PFO was not cytotoxic according to ISO 10993-5 (2009),[15] which indicated a cytotoxicity threshold of >30% cell mortality. When the cytotoxic control was applied on 59% and 83% areas, cell mortality equal or superior to 84% was observed in BALB/3T3 and ARPE-19 cells at all tested contact times, corresponding to cytotoxicity according to ISO 10993-5 (2009).[15] The not cytotoxic control applied on 59% and 83% area of ARPE-19 cells for 24 hours showed 13% and 17% mortality. When 59% and 83% contact areas were tested, ARPE-19 cells showed significantly higher cell mortality than BALB/3T3 cells at 24 hours contact time (Fig. 2; $P \leq 0.05$, Student's t-test).

The use of MTT and NRU assays for quantitative evaluation of cell mortality induced by 15, 50, and 80 µL of 1H PFO and PFO for 24 hours showed similar results in the ARPE-19 cell line (Table 4). The difference in percent cell mortality was not statistically significant under all tested conditions.

Figure 3 shows TUNEL assay pictures obtained under and around the specimen after application of not cytotoxic control (PFO; Figs. 3a–c) and cytotoxic control (12.5% 1H PFO; Figs. 3d–f). As observed by inverted microscopy at ×40 magnification, ARPE-19 cells were uniformly distributed after application of PFO with presence of sporadic apoptotic cells stained in blue (Fig. 3a) while in the cells incubated with cytotoxic control (1H PFO) the contact area showed the presence of apoptotic cells with altered morphology stained in blue (Figs. 3d, 3e).

Table 5 reports the mean apoptosis grading score obtained for each tested condition. Cells treated with not cytotoxic control (PFO) showed a grade of <1 under and out of the specimen as well as vehicle. Cells treated with cytotoxic control (12.5% 1H PFO) showed a grade of <1 out of the specimen and a grade of 3 under the specimen, corresponding to the presence of blue-stained apoptotic cells with altered morphology or acellular zones.

Both donor retina ex vivo and cell line models showed comparable cytotoxicity results. Not cytotox-



	NOT CYTOTOXIC CONTROL (PFO 99.8%)						CYTOTOXIC CONTROL (1H PFO 98.8%)					
	22% area		59% area		83% area		22% area		59% area		83% area	
	BALB3T3	ARPE19	BALB3T3	ARPE19	BALB3T3	ARPE19	BALB3T3	ARPE19	BALB3T3	ARPE19	BALB3T3	ARPE19
2.5 h contact time	-1,1	6,0	0,9	3,1	0,4	2,8	22,9	25,4	86,3	83,8	95,0	89,7
12 h contact time	2,9	0,9	4,5	6,4	5,7	10,5	14,0	23,1	93,3	93,1	99,0	97,0
24 h contact time	4,8	6,4	8,1	13,1	10,2	17,3	21,3	26,3	90,3	96,0	86,0	96,6

Figure 2. Quantitative viability evaluation. The percentage of cell mortality induced by cytotoxic and not cytotoxic controls under 22%, 59%, and 83% contact areas and 2.5, 12, and 24 hours of contact time conditions as measured by the MTT and NRU quantitative assays in ARPE-19 and BALB/3T3 cells, respectively. *Dashed line* indicates cytotoxicity threshold according to ISO 10993-5 (2009).

ic control (PFO) was confirmed as not cytotoxic in both models and showed similar percent cell viability (Table 6). Cytotoxic control sample (12.5% 1H PFO) was confirmed to be cytotoxic in both models. The percentage of cell viability in ARPE-19 cell line was significantly higher than that in the donor retina ex vivo model ($P < 0.05$). Based on the cell viability assay, the ARPE-19 cell line showed %CV from 7 to 11 and the %CV in donor retina ex vivo model ranged from 25 to 29 indicating "good" and "acceptable" method repeatability, respectively.

Discussion

The cytotoxicity test in vitro[15] aims at detecting potential toxicity of medical devices. Physicochemical analytical techniques, such as gas chromatography/mass spectroscopy, and UV absorption spectroscopy, detect and quantify the toxic impurities in PFCLs.[7] However, they do not directly demonstrate the toxicity of such impurities. The cytotoxicity test,[15] performed in addition to physicochemical analyses, assesses the cytotoxicity of total impurities present in

Table 4. The Percentage of ARPE-19 Cell Mortality Induced by Cytotoxic and Not Cytotoxic Controls After 24 Hours Contact Time as Measured by the MTT and NRU Quantitative Assays

Sample	Volume, μL	NRU Assay, % of ARPE-19 Cell Mortality	MTT Assay, % of ARPE-19 Cell Mortality	*P* Value, Student's *t*-test
Not cytotoxic control	15	8.2 ± 0.7	5.8 ± 2.7	0.66
	50	19.1 ± 0.2	12.9 ± 1.9	0.16
	80	18.4 ± 0.5	16.0 ± 1.9	0.52
Cytotoxic control	15	36.2 ± 0.3	29.9 ± 2.3	0.31
	50	97.8 ± 0.7	97.5 ± 1.0	0.70
	80	96.8 ± 0.1	97.3 ± 0.4	0.14

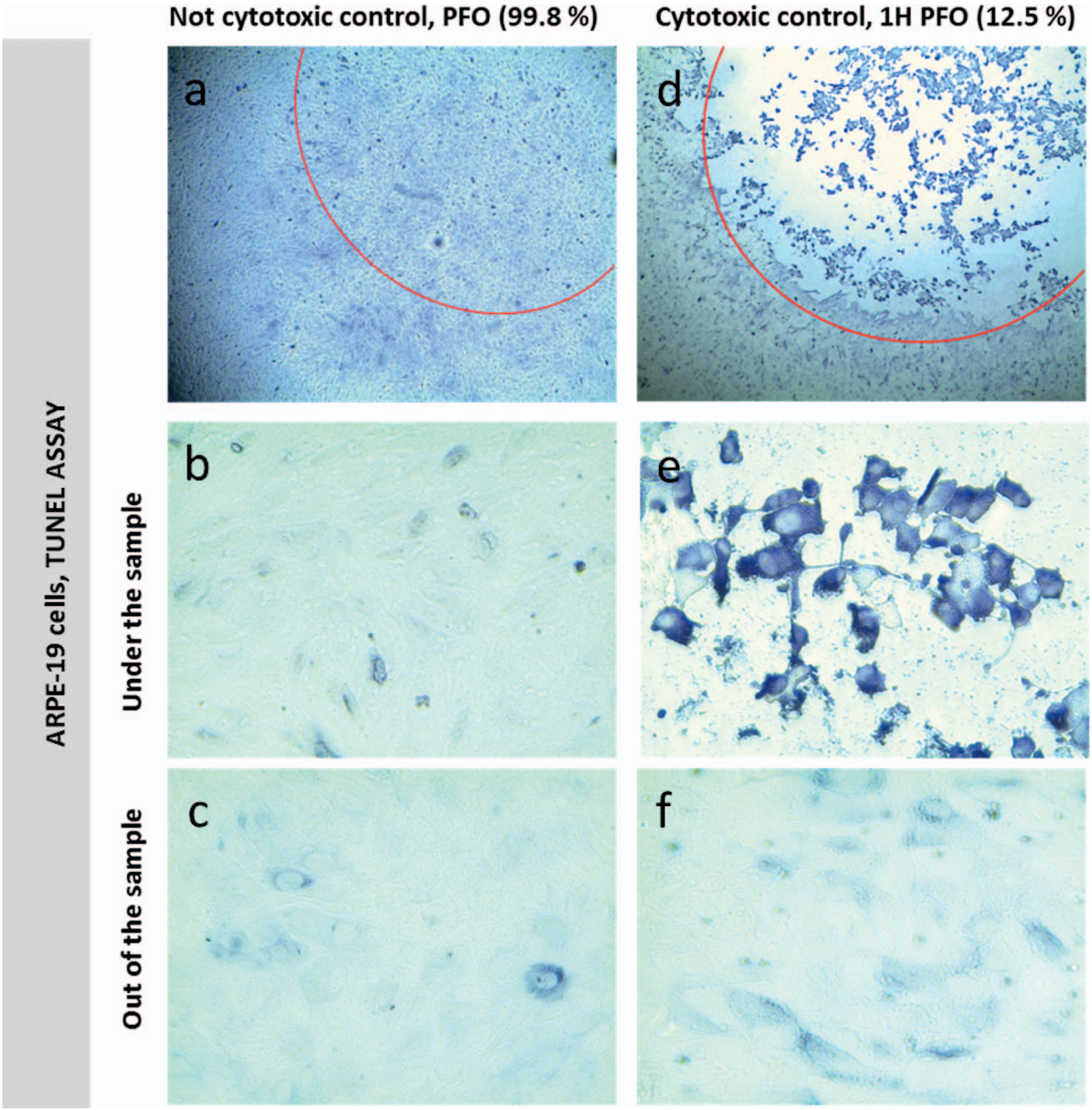


Figure 3. Qualitative evaluation of apoptosis by TUNEL assay. Light microscopy pictures at ×40 magnification of the ARPE-19 cells after not cytotoxic control (PFO) application for 24 hours (a), normal unstained cells under the PFO sample at ×400 magnification (b) and normal unstained cells out of the sample at ×400 magnification (c); cytotoxic control (1H PFO contact area, at ×40 magnification (d), blue-stained apoptotic cells under the cytotoxic control sample at ×400 magnification (e) and unstained cells with normal morphology out of the cytotoxic control sample at ×400 magnification (f). The borders of the sample contact area are indicated with *red lines*.

the sample. Therefore, it is advisable to perform chemical analyses during the selection of the raw materials in medical device manufacturing processes and cytotoxicity test on the finished product.

The manufacturer has the responsibility to choose the most appropriate tests based on device characteristics and intended use, and the method must be validated.[15] In our validation study, the direct contact

Table 5. Mean Grading Score ± SEM Obtained for Vehicle, Cytotoxic, and Not Cytotoxic Controls After TUNEL Assay

ARPE-19 Cell Line, 59%, 24 hours Contact (mean ± se)				
	Cytotoxic Control 12.5% 1H-PFO		Not Cytotoxic Control 99.8% PFO	
Vehicle	Under Specimen	Out of Specimen	Under Specimen	Out of Specimen
0.3 ± 0.1	3.0 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

test was selected as the most suitable option to determine the possible toxicologic hazard of PFCLs, because it simulates the clinical use conditions as a contact between the PFCL and the retina occurs during the vitreoretinal surgery. The indirect contact test was considered inappropriate because no contact is established between the sample and the cells and PFCLs would not diffuse through the agar layer used in the test. Similarly, the extract test does not simulate nor exaggerate the clinical use as requested by ISO 10993-5 (2009).[15] Moreover, the extraction of any toxic substances from PFCLs is a challenging procedure because of the high volatility and immiscibility of PFCLs with aqueous and organic solvents. As noted also by Pastor et al.[18] the weakness and unreliability of the extract test performed by the manufacturer of PFO Ala-Octa can be found in the same ISO guidelines.[15] The inadequacy of the extract test has been demonstrated clearly, failing to prove the cytotoxicity of raw PFO, when performed by Alamedics, and of the toxic PFO lots, when performed by IOBA.[18,19]

Considering the particular chemical and physical specifications of PFCL, we assessed the risks of possible direct contact test failure to point out the critical phases of the test to address them in the validation study. The main risks corresponded to the sample evaporation during testing, insufficient contact area and contact time between the sample and the cell layer, cell mortality induced by sample heaviness, and inappropriate cell sensibility. First, we established and described a method for PFCLs direct deposition on the cell layer to guarantee the direct contact between the PFCL samples and the cell layer for the whole duration of the test and to avoid sample evaporation or loss of PFCL contact with the cell layer (Supplementary Movie S1). We deposited the PFO bubble on the cell layer in the presence of the medium to avoid PFO evaporation and assure that the sample remained in contact with the cells. Conversely, PFO samples were placed on the cell layer before adding the culture medium in other studies.[18,19] Second, according to the ISO 10993-5 (2009),[15] the contact area between the sample and cell layer should correspond to one-tenth of the cell layer surface. We tested three contact areas and we showed that the contact area of 22% failed to detect the toxicity of 1H PFO by a quantitative approach in both tested cell lines. This was mainly because 1H PFO induced the cell death in the area located directly

Table 6. Cytotoxicity Test Results, Cell Viability ± SEM (%), %CV, and *P* Values of Mann-Whitney *U* Test in Retinal Tissues and ARPE-19 Cell Line

Parameter	Donor Retina Ex Vivo Ø3 mm, $n = 47$	ARPE-19 Cell Line, $n = 48$	*P* Value
Not cytotoxic control, PFO (99.8 %)			
Cytotoxicity (cell viability <70%[15])	Not cytotoxic	Not cytotoxic	n.a.
Cell viability (% ± SE)	92 ± 3	97 ± 1	0.104
CV (% ± SE)	29	7	n.a.
Cytotoxic control 12.5% 1H-PFO in PFO			
Cytotoxicity (cell viability < 70%[15])	Cytotoxic	Cytotoxic	n.a.
cell viability (% ± SE)	25 ± 1	64 ± 1	0.000*
CV (% ± SE)	25	11	n.a.

n.a., not applicable.
* $P < 0.05$ according to Mann-Whitney *U* test.

under the sample and all the cells located out of the 22% contact area remained viable and the percent of cell mortality was roughly proportional to the percent of contact area, which was under the 30% cytotoxicity threshold indicated by the ISO 10993-5 (2009).[15] The cytotoxicity was clearly detected when increased (59% and 83%) contact areas were tested by the quantitative approach.

Ultrapure PFO induced slight cell mortality in ARPE-19 cells, which increased proportionally with the contact area and contact time. We hypothesize that the mortality was induced by the excessive pressure on the cell layer exerted by the sample weight.

Considering that the 83% contact area induced slight mortality due to the sample heaviness, and 22% area was insufficient to detect cytotoxicity of 1H PFO, we selected the 59% contact area as an appropriate contact area for the cytotoxicity test of PFCLs. Third, for each tested contact area, we assessed three contact times (2.5, 12, and 24 hours) between the sample and the cells, because different contact times may determine different cytotoxicity values. Even if the ISO 10993-5 (2009)[15] indication is to use 24 hours contact time, Pastor et al.[18] and Srivastava et al.[19] selected 30- and 60-minute time intervals motivating this choice with the simulation of PFO clinical use. Our study showed that all tested time conditions, including 2.5 hours contact time, could reliably detect the cytotoxicity only when the contact area corresponded to at least 59% of the cell layer. As mentioned before, for smaller contact area (22%), the toxicity of 1H PFO was not detected by quantitative approach at any tested time interval. This indicated that the contact area is a more critical factor compared to the contact time in the cytotoxicity test of PFCL by direct contact test. The contact areas were not described by previous studies[18,19] which reported to test 80 µL volume that in our study corresponded to 83% contact area.

We confirmed 24 hours contact time, as indicated by ISO 10993-5 (2009),[15] as an appropriate contact time interval to detect the cytotoxicity of PFCLs. We performed a comparison of the sensitivity of the ARPE-19 and BALB/3T3 because the literature reported the use of different cell lines.[18,19] ARPE-19 cells showed significantly higher percentage of cell mortality compared to BALB/3T3 cells by the direct contact test quantitative evaluation when 59% and 83% contact areas were tested at a 24-hour time interval. Consequently, we selected the ARPE-19 cells as the most appropriate cell line because it mimics the cells in clinical use and it was more sensitive in detecting the PFCL cytotoxicity. Our data confirmed equivalence between NRU and MTT assays for ARPE-19 cells.

The optimal cytotoxic control should be similar to the test sample and give reproducible cytotoxic response corresponding to 50% cell mortality.[15] In our study, this was obtained by the use of diluted 1H PFO (12.5% 1H PFO in pure PFO) applied on the 59% contact area of ARPE-19 cells for 24 hours (not shown). In previous studies,[18,19] the liquefied phenol was used as the cytotoxic control. The percentage of the viability response was not reported by the authors, and physicochemical properties of liquefied phenol differ substantially from PFCL samples in terms of miscibility, volatility, density, and sample preparation deposition and removal; therefore, this option was not considered in our validation study.

Finally, we evaluated cell viability also in a human retina ex vivo culture model and compared it to the cytotoxicity test performed in the ARPE-19 cell line. Both models detected correctly 12.5% 1H PFO as cytotoxic and PFO as not cytotoxic based on 30% cell viability reduction threshold.[15] The percentage of cell viability was comparable in the two models after application of not cytotoxic control, whereas cytotoxic control (12.5% 1H PFO) induced higher percent cell mortality in a human retina ex vivo model, indicating its higher sensibility. Both models showed acceptable repeatability parameters with better %CV for the ARPE-19 cell line. Pastor et al.[18] reported that the cytotoxicity results obtained with cell cultures were confirmed by the qualitative examination of a porcine neuroretina explants model[19] in which, however, the quantitative cell viability has not been determined.[19]

In our study the qualitative evaluation of the cells was performed based on the grading score in the zone immediately under and around the tested sample before and after NRU and MTT vitality assays. A similar qualitative approach was used for evaluation of apoptosis by the TUNEL assay. Assessment of cells under the PFCL samples in the qualitative evaluation was critical and showed clear cytotoxicity and apoptosis in the contact area, whereas no cytotoxicity and no apoptosis were detected in the rest of the cell layer. This indicated the absence of diffusion of cytotoxic control sample from the sample bubble after 24 hours of application. Thus, the qualitative evaluation clearly pointed out the toxicity of 1H PFO in all contact areas and time conditions, differently from the quantitative approach where

detection of the toxicity failed for the 22% contact area. Moreover, the qualitative evaluation allowed to observe that the morphology of the ARPE-19 cells evidently was not altered in the presence of the heavy not cytotoxic control samples, indicating that the ARPE-19 cells were possibly more resistant to the pressure damage compared to the BALB/3T3 cells.

In conclusion, our study confirmed the adequacy of the ISO 10993-5 (2009)[15] standard as a direct contact cytotoxicity test for PFCL samples, which was validated using qualitative and quantitative approaches in ARPE-19 cells, BALB/3T3 cells, and a qualitative human retina ex vivo model with 59% of contact area and 24 hours of contact time, and the use of 12.5% 1H PFO and 99.8% PFO as cytotoxic and not cytotoxic controls, respectively.

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