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CLINICAL INVESTIGATION

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Effects of different perfluorochemicals on dorsal root ganglion cells in vitro

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Abstract ● Background: Investigation of the effects of different perfluorochemicals (PFC) on cultured dorsal root ganglion (DRG) cells. ● Method: DRG cell cultures from 9- to 11-day-old chicken embryos were exposed to emulsified perfluorodecalin (PFD; $C_{10}F_{18}$; 0.5%, 1% and 10%) or perfluorooctylbromide (PFO; $C_8F_{17}Br$; 0.5%, 1% and 10%). The cells were evaluated under phase-contrast optics after 30 h and 120 h for 0.5 and 1% and after 5 h for 10%. To study the integrity of neuronal cells, immunohistochemical labelling for neurofilaments (NF) and tubulin (TUB) was performed. ● Results: Concentrations of 0.5% and 1% of PFD or PFO did not change immunohistochemical labelling of DRG cells. Co-cultured macrophages showed a foam cell response, presumably representing ingested PFC. At both concentrations PFD induced a weaker foam cell response than PFO. A concentration of 10% led to the death of DRG cells and macrophages within 5 h. ● Conclusion: PFC caused a dose-dependent damage of neuronal cells. Co-cultured macrophages developed a foam cell response similar to that observed in vivo after prolonged presence of PFC in the vitreous body. These observations indicate that PFD and PFO may not be suitable for long-term vitreous replacement in vitreoretinal surgery. However, the model is limited by several factors: (1) there are physiological differences between DRG cells and retinal ganglion cells; (2) in vivo retinal ganglion cells are protected by the overlying tissues; (3) the PFC used in tissue culture must be emulsified.

Introduction

Because of their high specific weight, perfluorochemicals (PFC) are used in vitreoretinal surgery for complicated retinal detachments to facilitate reattachment of the retina [3, 5, 8]. The brief presence of perfluorochemicals in the vitreous body is well tolerated [5, 6, 10, 18, 23]. However, prolonged intraocular tamponade with PFC has been reported to cause problems. The side effects after long-term vitreous tamponade with PFC include liquid dispersion, growth of epiretinal membranes, tractional retinal detachment, degenerative alterations of the photoreceptors and activation of macrophages in the retina, the so-called foam cell response [4, 6, 8–10, 16, 23]. To assess the effects of perfluorodecalin (PFD) and perfluorooctylbromide (PFO) on ganglion cells, cultured dorsal root ganglion cells were selected as an in vitro model.

Materials and methods

Cell culture

Dorsal root ganglion (DRG) cells from 9- to 11-day-old chicken embryos were dissected in Hank's solution, transferred into prewarmed (37° C) dissociation medium (0.05% trypsin, 1:250 in calcium-magnesium-free saline) and then stirred gently with a Teflon-covered magnetic stirring bar. After 5 min agitation, the super-

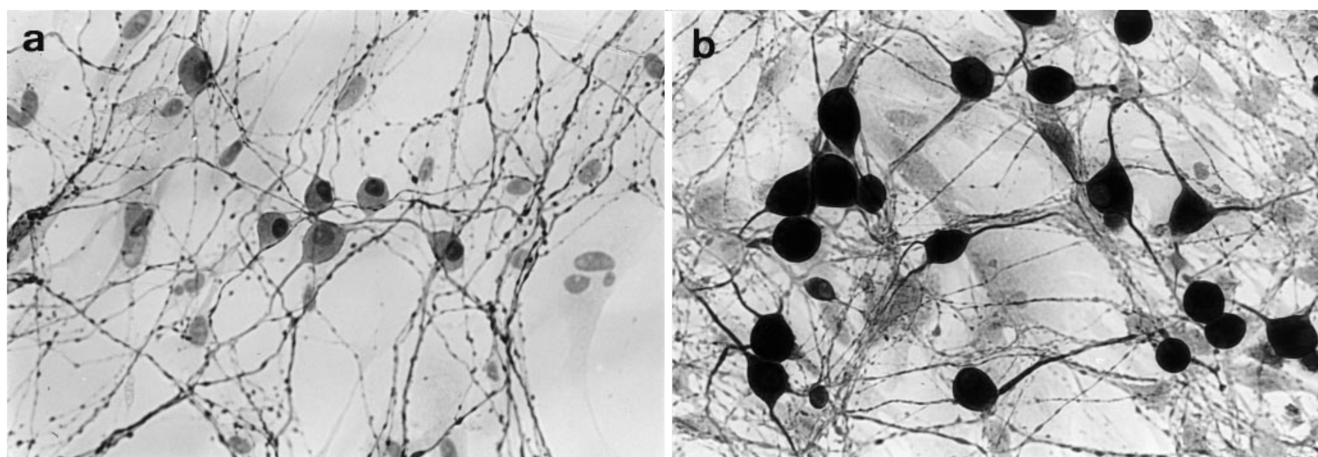

Fig. 1 **a** Immunohistochemical labelling of untreated DRG cells with the monoclonal antibody directed against NF. The processes of DRG cells exhibited a beaded labelling with NF. **b** Immunohistochemical labelling of untreated DRG cells with the antibody directed against TUB. Magnification ×210

natant containing the dissociated cells was transferred into a centrifuge tube containing 20 ml minimal essential medium (MEM), 1% chicken embryo extract, 6% glucose and 10% horse serum. After the addition of fresh trypsin solution (37° C), the DRG cell material was gently stirred for another 5 min. This procedure was repeated four times.The suspension containing the collected cells was centrifuged for 5 min at 900 *g*. The cell pellet was then resuspended in 4 ml fresh nutrient medium. Aliquots of one drop ($\sim 1 \times 10^5$ cells/ml) of this resuspension were cultivated for 1–2 weeks on glass coverslips and coated with rat-tail collagen in an incubator (5% CO_2, 37° C, 90% humidity) [15].

Experiments

Three to six DRG cell cultures for each group were exposed to emulsified and highly purified (minimum purity of 99.9%) PFD ($C_{10}F_{18}$) or PFO ($C_8F_{17}Br$). The PFD emulsion was received from the University of Ulm and consisted of 19% (w/v) perfluorodecalin, 0.5% egg lecithin, 2.5% synperonic F68 and 0.5% (w/v) perfluoroperhydrophenanthren as surfactants, with particle size between 0.18 and 0.25 μm. The PFO emulsion was received from the University of Nice, France, and consisted of 90% (w/v) perfluorooctylbromide and 4% egg lecithin. The mean particle size was 0.25 μm. The emulsifiers egg lecithin and synperonic F68 were tested separately for toxicity by the same culture model (see below). Both emulsions were stable at 5° C for more than 4 months. Stability tests were performed by particle size analysis.

In preliminary experiments using undiluted emulsified PFC, the DRG cells were destroyed within less than 1 h. In these experiments the DRG cell monolayer was completely superimposed by PFC. However, to show that the results obtained are not an artifact of lacking nutrient agent additional experiments (n=4) using NaCl (0.9%) without nutrient medium were performed. Under these conditions the cells showed no alteration after 1 h.

Therefore, PFC emulsions were diluted in MEM to the actual concentration of 10%, 1%, and 0.5%. Cultures exposed to 0.5 and 1% PFC were investigated after 30 h and 120 h, cultures exposed to 10% after 5 h. For this purpose the cultures were washed in phosphate-buffered saline (PBS; Sigma, Germany), fixed in 4% paraformaldehyde for 30 min, and evaluated under an inverse microscope and phase-contrast optics (Zeiss, Germany). Untreated cell cultures (n=39) were used as controls. The medium was not changed during the experiments.

Emulsifiers

Egg lecithin and synperonic F68 were tested separately for toxicity using the same culture model. Morphologic changes on DRG cell cultures were observed with undiluted emulsified PFC and 10% diluted emulsified PFC after 1 h and 5 h, respectively (see Results). To exclude a possible toxic effect at these concentrations and over these critical exposure times by the emulsifiers, the DRG cell cultures were tested separately over 1 h with 4% egg lecithin (n=4) or 2.5% synperonic F68 (n=4) and over 5 h with 0.44% egg lecithin (n=4) or 1.3% synperonic F68 (n=4).

Immunohistochemistry

Immunohistochemical labelling for neurofilaments (NF) and tubulin (TUB) was performed by the peroxidase-antiperoxidase (PAP) method [21] using mouse monoclonal antibody anti-NF 200 kDa (clone NF 14, Boehringer Mannheim, Germany) and rabbit polyclonal anti-TUB (Sigma, Germany). The monoclonal antibody to NF 200 kDa is directed against the phosphorylated epitope of NF 200 kDa. After exposure to 1% Triton in PBS at pH 7.6 for 10 min, the cultures were treated with 3% H_2O_2 for 20 min to inactivate endogenous peroxidase. The cultures were then incubated with anti-TUB (1:40 in PBS) and anti-NF (1:20) for 1 h. After washing in PBS, normal goat serum (1:50) was used for 30 min to block unspecific binding sites. The cell cultures were then treated with goat-anti-mouse IgG (or goat-anti-rabbit IgG for the polyclonal antibody directed against TUB, each diluted 1:50 in PBS) and then washed with PBS. Finally, the cell cultures were reacted with mouse PAP complex (or rabbit PAP complex for the polyclonal antibody directed against TUB, each diluted 1:50 in PBS). The peroxidase was visualized using 0.06% diaminobenzidine as chromogen and 0.3% H_2O_2 in phosphate buffer at pH 7.6. Omission of the first antibody was used as a negative control.

Results

Controls

Under control conditions DRG cells showed marked immunoreactivity with the antibodies against NF (Fig. 1a)

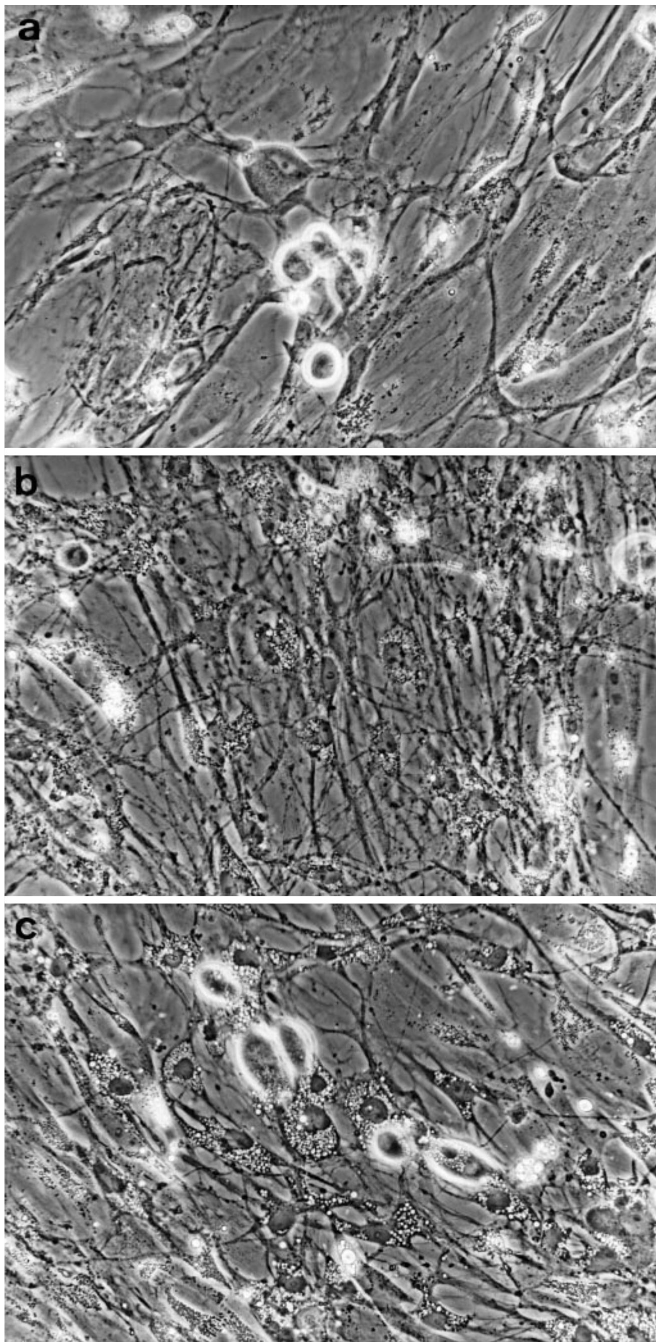

Fig. 2 **a** Control cell culture showing DRG cells with co-cultured macrophages. **b** Foam cell response of co-cultured macrophages at a concentration of 0.5% PFD. **c** Foam cell response of co-cultured macrophages at a concentration of 0.5% PFO. Magnification ×400

and TUB (Fig. 1b). TUB showed stronger immunohistochemical labelling in the somata than NF, which prevailed in the processes, where it exhibited beaded labelling.

Emulsifiers

The emulsifiers egg lecithin and synperonic F68 were tested separately with the same culture model and showed no effect on DRG cells compared to non-treated controls. Immunohistochemical labelling for neurofilaments remained unchanged in these experiments.

Perfluorochemicals

After 30 h, co-cultured macrophages treated with 0.5% PFD or PFO showed, compared to non-treated controls (Fig. 2a), phagocytosis of PFD (Fig. 2b) and PFO (Fig. 2c); this phagocytosis was more pronounced after a period of 120 h. The immunohistochemical labelling for NF and TUB in the DRG cells was unchanged compared to the non-treated controls.

Co-cultured macrophages treated with 1% PFD or PFO showed increased phagocytosis, which was less pronounced in cells treated with PFD than in those treated with PFO. The immunohistochemical labelling of the antibodies against NF and TUB was not changed after 30 h and 120 h exposure, respectively.

Application of 10% PFD or PFO to the DRG cells led to degenerative alterations within 5 h. The number of DRG cells and macrophages was markedly reduced with PFD, causing fewer pronounced degenerative alterations than PFO. The remaining neurons revealed severe shrinkage of both somata and processes in most cases and pathological swelling in some others. The immunohistochemical labelling for NF and TUB was strongly diminished as a result of progressive cell loss (Figs. 1, 3). The remaining neurons presented increased immunoreactivity for NF in the perikaryon and in the proximal neurite stump (Figs. 3a, b). Furthermore, NF-labelled neurons sporadically showed degenerative characteristics such as swelling of the somata and processes (Fig. 3b). In contrast to cells under control conditions, in processes of individual neurons TUB showed multiple swelling in sequence (Fig. 3c). Both for NF and TUB, the network of processes had been distinctly changed (Figs. 3b, d). Various degenerative manifestations were detected in macrophages.

Discussion

In this study, cell cultures of dorsal root ganglia were employed to validate toxicity of PFC. PFC caused dose-dependent effects on neuronal cell cultures.

Neurofilaments and tubulin

Neurofilaments and tubulin are sensitive neuronal structure proteins [11, 19, 20]. They define cellular integrity

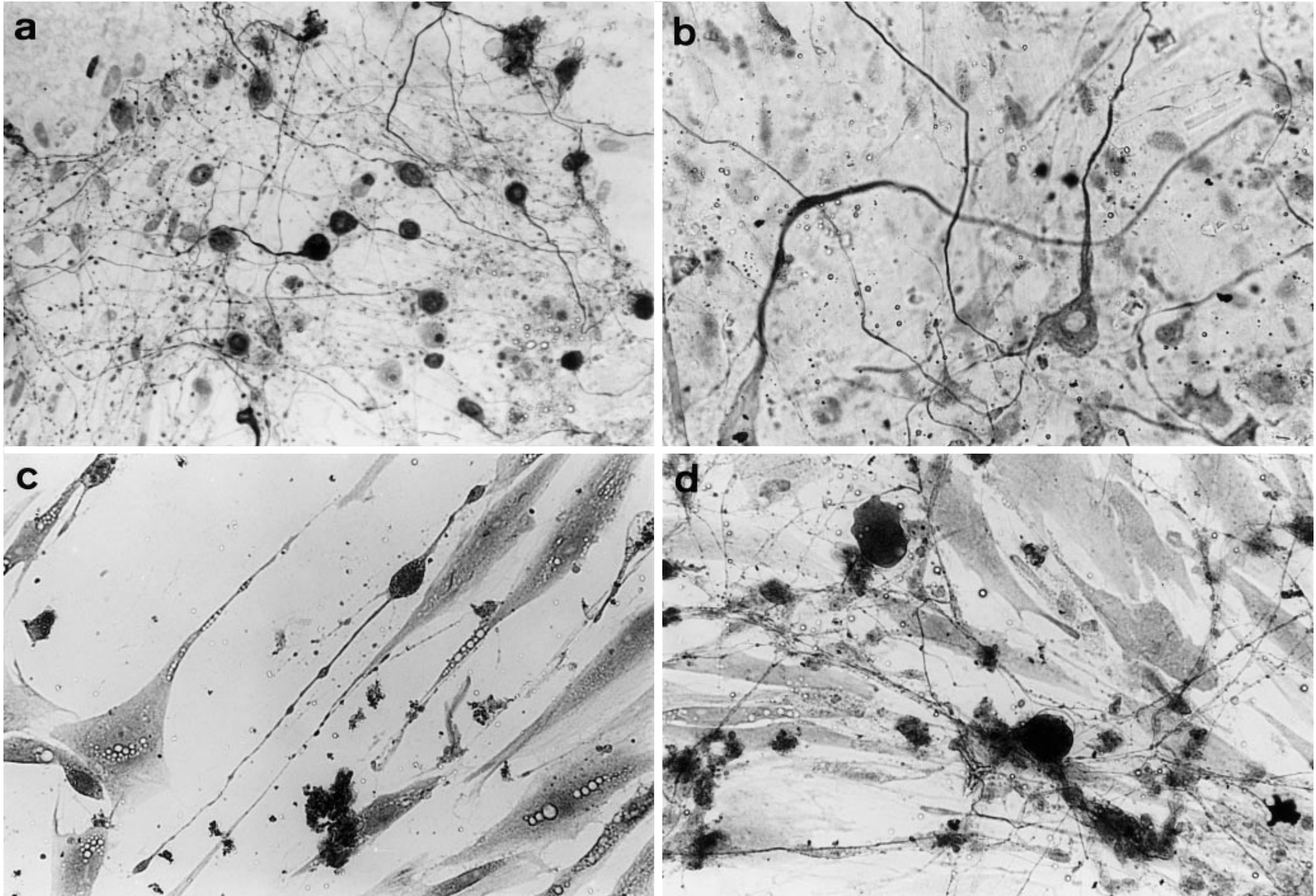

Fig. 3 **a** Immunohistochemical labelling of DRG cells treated with 10% PFD with the antibody directed against NF. Cell bodies of surviving DRG cells revealed enhanced labelling for NF compared to controls. **b** Immunohistochemical labelling of DRG cells treated with 10% PFO with the antibody directed against NF. **c** Immunohistochemical labelling of DRG cells treated with 10% PFD with the antibody directed against TUB. TUB showed beaded fibers and club formation at the distal part of the remaining processes. **d** Immunohistochemical labelling of DRG cells treated with 10% PFO with the antibody directed against TUB. The immunolabelling of TUB was markedly reduced compared to controls. Magnification: a, b, d ×210; c ×260

via the neuronal cytoskeleton. The cell cultures treated with PFC were therefore tested immunohistochemically with antibodies against NF and TUB for changes in the neuronal cytoskeleton.

In altered neurons the monoclonal antibody directed against the phosphorylated epitope of NF 200 kDa showed increased immunoreactivity in the somata and the proximal part of the processes. Similar results were observed in the retinal ganglion cells following axotomy of the optic nerve [7]. Disturbed axoplasmic transport and posttranslational modification of NF are discussed as possible causes for this phenomenon [13, 17, 22].

TUB showed club-shaped distensions of the distal part of the processes of the degenerating neurons. Similar axonal changes observed with antibodies directed against various epitopes of NF have been described in experimental lesions in the visual cortex [14]. In the sequence of axonal damage, the proximal stump, retaining residually functioning axoplasmic transport, shows an accumulation of axoplasmic organelles that leads to club-shaped distensions of the proximal stump end [12]. The effects observed differed with the various PFC used for testing. PFD showed fewer cell alterations than PFO, although both were used in identical concentrations and allowed to react for the same period of time. Magnetometric testing also revealed dose- and substance-dependent effects of PFC [2].

Foam cell response

Our experiment confirms the foam cell response observed in animal tests [6, 10, 16, 23]. As postulated by Augustin et al. [1, 2], macrophage alteration caused by ingested PFC can advance the propagation of the damage. Accordingly, an increased foam cell response entails increased potential toxicity. In this study, primary degenerative phe-

nomena among the macrophages were observed with 10% concentrations of PFD or PFO, as was done by magnetometry [2]. Thus, both techniques are a sensitive means of identifying PFC-induced foam cell response.

Toxicity of perfluorochemicals

In this test series, emulsified PFD and PFO were both shown to alter neuronal cell populations after brief exposure. A toxic effect at the critical corresponding concentrations and exposure times by the emulsifiers, could be excluded by using the same culture model. Hence, the tested PFC appear rather unsuitable for long-term tamponade, especially in cases where the ganglion cells are not protected by overlying tissue. Possible contact with the neuronal elements of the retina must be considered when PFC infiltrate the subretinal area as an intraoperative complication in vitrectomies of complex retinal detachment [8]. Moreover, Eckardt et al. [10] hypothesized the infiltration of PFC into the entire retina as a further pathophysiological mechanism for retinal toxicity of PFC.

Other experimental and clinical test series have described the unsuitability of PFC for long-term tamponade [4, 6, 9, 10, 24]. This was made evident by such manifestations as alterations of the photoreceptors and the outer nuclear and ganglion cell layers with subsequent atrophy of the retina [9, 10, 23]. A mechanical lesion caused by PFC with subsequent degeneration of retinal elements discussed in preliminary studies cannot be excluded in the present study. However, it seems rather improbable considering the concentrations of 0.5–10% used herein [4, 6, 8, 10, 23]. Accordingly, the observation that retinal necrosis occurs with intravitreal tamponade with various PFC at the perfluorochemical liquid-aqueous interface [23] leads to the assumption that other pathophysiological mechanisms must be involved in addition to the purely mechanical alteration of the retina evoked by PFC.

A model always differs from the real conditions. Thus, there was no equivalent of the inner limiting membrane in the culture, and the arrangement of the neural elements within the nerve fiber layer and the ganglion cell layer in the living eye is certainly different than the monostratified random distribution encountered under tissue culture conditions. We also cannot be certain that immature cells from chicken embryos are more sensitive that mature ganglion cells in the human retina.

On the other hand, we have proven with our experiments that 5 h of exposure to 10% PFC emulsions has a disastrous effect on some neural tissue. Based on this observation, it seems justified at least to issue a word of warning to those considering placing 100% PFC in contact with the retinal surface for prolonged periods of time. Despite the limitations of this model, cell culture investigations combined with immunohistochemistry represent an additional experimental tool for the prediction of PFC toxicity.

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