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THE CYTOTOXICITY IN VERO CELLS OF A PERFLUOROCARBON USED IN VITREORETINAL SURGERY

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ABSTRACT

Perfluoro-n-octane is a high-density liquid perfluorocarbon used as a long-term vitreous replacement in vitreoretinal surgery. In this study, we assessed the toxicity of perfluoro-n-octane (PFOC) on Vero cells using an indirect toxicity test, scanning electron microscopy and immunocytochemistry. For indirect toxicity test, Vero cells were cultured for 12 h with PFOC extracts in 96-well plates and the plates were, then, read at 540 nm. For direct toxicity, Vero cells were incubated with 0.1 ml of perfluoro-n-octane alone. Glass coverslips were used as inert control for weight, to produce mechanical compression similar in weight and area to that caused by perfluoro-n-octane. Cells cultured without PFOC were used as a negative control. Silicone bands with a weight and area similar to those of perfluoro-n-octane served as a positive control for toxicity. The indirect toxicity test showed that perfluoro-n-octane did not release soluble toxic substances that affected cell growth. In the direct toxicity test, the cells in the control group had homogenously distributed actin filaments, although scanning electron microscopy revealed some vesicles on the cell surface. In the controls for weight, cytoplasmic retraction and the formation of thin cellular prolongations were seen. Cells incubated with perfluoro-n-octane showed greater changes compared to those seen in cells under a similar control weight. Silicone-treated cells had an irregular or fragmented morphology. These results show that, in addition to its compressive action on cultured cells, perfluoro-n-octane may also exert a toxic effect.

Key words: Perfluorocarbon liquids, perfluoroctane, Vero cells, cytotoxicity

INTRODUCTION

Vitreoretinal surgery is a complex procedure which often has a less positive functional and anatomical outcome than other eye surgeries because it is not always efficient in maintaining the retina reattached after surgery. Depending on the size of the tear and extension of the detachment, fibrous proliferation may occur and keep the retina detached. The use of perfluorocarbon liquids (PFCLs), which are synthetic compounds with fluoro-carbon bonds, was introduced in ophthalmology in an attempt to reattach the retina by using more effective and less traumatic methods. PFCLs with physical properties such as immiscibility with water and blood [16], optical transparency and a high specific weight (increased weight relative to the same volume), became hydrokinetic tools for stabilizing the retina in the posterior pole of the eye during surgery [3,14]. These substances have a low viscosity, thus facilitating their insertion into and removal from the eye. Ideally, the liquid should remain in the eye for some time after surgery in order to guarantee anatomic reapposition and attachment of the retina [18].

Perfluoro-n-octane is the most widely used PFCL in ophthalmology. There is some controversy in the literature about the toxicity of perfluorocarbons. Pure PFCLs are usually considered inert. The toxic effects of PFCLs may be caused by impurities with nitrogen, hydrogen on unsaturated carbon bonds [16]. Small amounts of residual perfluorocarbon in the vitreous humor after its intraoperative use are not toxic [7]. Histopathological evaluation has shown that treatments with intravitreal drops (0.1 ml) of perfluorocarbons is well tolerated for up to six months [4].

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Studies have suggested that PFCLs retained postoperatively in patients' eyes may act as buffering agent and may cause inflammatory reactions and cellular injury involving compression, structural disarrangement of the retina, photoreceptor damage, the accumulation of macrophages in the retina, and a reduction in the number of cells [4,13]. However, it is unclear whether these effects result from toxicity or mechanical injury. Progressive damage proportional to the length of time the retina was in contact with this substance has been reported [13,20].

In a review of the uses of PFCLs, Peyman *et al*. [16] pointed out that toxicity might have a physical rather than chemical nature. Liang and Peyman [11] assessed the long-term tolerance to a mixture in which different concentrations of perfluorocarbon liquids, perfluoro-n-octane, and perfluoroperhydrophenanthrene were used in rabbit eyes, and found no toxic effects. In addition, in cells cultured with perfluorocarbon liquids, injury resulted from compression by weight of the compound rather than through a toxic action [14]. Green *et al*. [7] reported that in rabbits the retention of more than 25% of perfluoro-n-octane (PFOC) in the vitreous cavity for up to seven weeks did not result in toxic effects.

In contrast, Eckardt *et al*. [5] assessed the intraocular tolerance of two PFCLs (perfluoro-n-octane and perfluoropolyether) and concluded that the histological alterations in the lower part of the retina indicated toxicity. Similarly, other studies have also shown that the use of highly purified PFCLs as long-term vitreous replacements can be toxic to the retina [2,20].

The use of cell cultures to assess the bioactivity and cytotoxicity of biomaterials has overcome some of the limitations of studies *in vivo*. The aim of this study was to examine the effect of perfluoro-n-octane (PFOC) in cultured Vero cells as these cells have been used to study cell growth and differentiation as well as interactions with biomaterials [8,6,17] and cytotoxicity [12], and are recommended for such investigations in standard protocols [1,9,10].

MATERIAL AND METHODS

Cell culture

Vero cells, a fibroblastic cell line established from renal cells of the African green monkey (*Cercophithecus aethiops),* were used. The cells were maintained in Ham's F10 medium (Sigma Chemical Co., St. Louis, MO, USA) with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37ºC.

Perfluoro-n-octane (PFOC):

The PFOC (Ophthalmos Ltda.) used in this study had the following chemical features: empirical and molecular formula = C_8F_{18}, molecular weight = 438, specific gravity = 1.76 g/ml, viscosity = 0.8 (centistoke, at 25ºC), refractive index = 1.27, and surface tension = 14 dyne/cm. The product (purity ~ 100%) was purchased in sterile form (5 ml bottles) suitable for medical use. When required, PFOC was added slowly to the culture medium to avoid the formation of bubbles.

Indirect cytotoxicity test

PFOC was added to the culture medium at a final concentration of 2 g/ml and then incubated at 37ºC for 48 h without shaking. After this period, the medium was harvested and the PFOC present was discarded. Using this approach, it was possible to assess the potential effect of substances released by PFOC into the culture medium. The indirect cytotoxicity test and the above extract were prepared and tested according to the ISO-10993 [9] and ASTM F813-83 [1] international standards.

Quantitative analysis of extracts in cultured cells

The method described by Murakami *et al.* [15] was used for quantitative analysis of the extracts in cultured cells. One hundred micro liters of a Vero cell suspension containing 1.0 x 10^6 cells/ml of Ham's F10 medium with 10% FCS were transferred to the wells of a 96-well culture plate (Corning Co., Cambridge, MA, USA) and cultured for 12 h at 37°C. After this incubation, the culture medium was removed and 100 µl of new medium containing the PFOC extract was added to each well. After 12 h at 37ºC, the extracts were removed and the cells were washed with 0.1 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.4 at 37ºC, then fixed in 10% formalin, washed in PBS and stained with crystal violet 0.05% (in 20% methanol). Before processing for quantitative analysis, the morphology of control and PFOC-treated cells was documented. The samples were then washed twice in PBS and incubated in 0.1 M sodium citrate (in 50% ethanol, pH 4.2) for 12 h. The plate with the remaining cells was then read at 540 nm in a Multiskan Bichromatic Version 1.06 microplate reader. An extract of culture plate material (propylene) was used as a negative control (no cytotoxic effect). A total of 16 samples were used in each experiment.

Direct cytotoxicity test

The direct cytotoxicity test consisted of assessing the possible toxic effects of PFOC in direct contact with Vero cells. A suspension containing 1 x 10^5 cells/ml of Ham's F10 medium with 10% FCS was inoculated 1 ml/well in a 24-well plate (Corning). The plates were incubated at 37°C for 12 h. After the incubation, 0.1 ml of PFOC was added on the cell layer. Glass coverslips were used as weight control to produce mechanical compression similar to the weight and area of the PFOC used. Cells cultured in

a PFOC-free medium and without coverslips were used as a negative control. Silicone adhesive membranes (provided by Dr. Eliana A. R. Duek, Department of Material Engineering, Faculty of Mechanical Engineering, Unicamp) was used as a positive control for cytotoxicity and had a weight and area proportional to those of PFOC. The samples were collected and fixed 12 h after the addition of PFOC. This contact time was established based on preliminary experiments (shorter times produced little change whereas longer times led to cell destruction and precluded any type of analysis). The cells in all treatments were analyzed morphologically and immunocytochemically.

To determine the area of the wells without cells in the different experimental conditions (an indicator of cell death), the cells were cultured as described above, fixed in Karnovsky solution, and stained with toluidine blue at pH 7. For cells incubated with square glass coverslips (negative control) or square silicone bands (positive control) the area was determined by multiplying the base by the height value. The PFOC area (circular) was determined by πr^2, where r is the radius of the circle. The measurements were made with calipers (n = 6).

Immunocytochemical analysis

Actin was detected immunocytochemically in cultured cells. After a 12 h incubation with the test agent, the culture medium was removed and the plates were washed with PBS at 37°C. The material was fixed in Karnovsky solution containing 0.2 % Triton X100 for 30 min, washed with PBS at 37°C and incubated with PBS containing 1% bovine serum albumin (BSA, Sigma) for 1 h at 4°C. The cells were incubated with primary anti-actin monoclonal antibody (1:200 dilution, Sigma, clone AL - 40) for 18 h at 4°C. After washing in PBS with 1% BSA, the cells were incubated for 1 h with a FITC-conjugated secondary anti-mouse IgG antibody (1:200 dilution, Sigma), then washed again with PBS and 1% BSA, before mounting in Vectashield. The cells were examined using an Olympus IX-50 inverted microscope fitted with fluorescence filters.

Scanning electron microscopy (SEM)

Cells cultured as described above were fixed in 2.5% paraformaldehyde/glutaraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.4, then washed in phosphate buffer followed by post-fixation with 1% osmium tetroxide (Sigma) and dehydration in an ethanol series. The cells were then critical point dried (Balzers CPDO030) and gold sputtered (Balzers 050) before being analyzed in a scanning electron microscope (Jeol JSM-5800 LV).

Statistical analysis

The results of the indirect and direct cytotoxicity test were analyzed using one-way ANOVA, with a significance level of $p < 0.05$.

RESULTS

Areas without cells in the different treatments

The areas without cells following incubation with PFOC, silicone and coverslips (the latter two being positive and negative controls, respectively) were 0.329 ± 0.088, 0.277 ± 0.005 and 0.401 ± 0.051 cm^2, respectively. There were no significant differences among these values.

Indirect cytotoxicity test

The absorbances at 540 nm were 0.306 ± 0.021 and 0.340 ± 0.030, for control and PFOC-treated cells, respectively. Thus, PFOC did not release soluble toxic substances into the culture medium. These findings agrees with the similar morphology of PFOC-treated and control cells seen following crystal violet staining (Fig. 1).

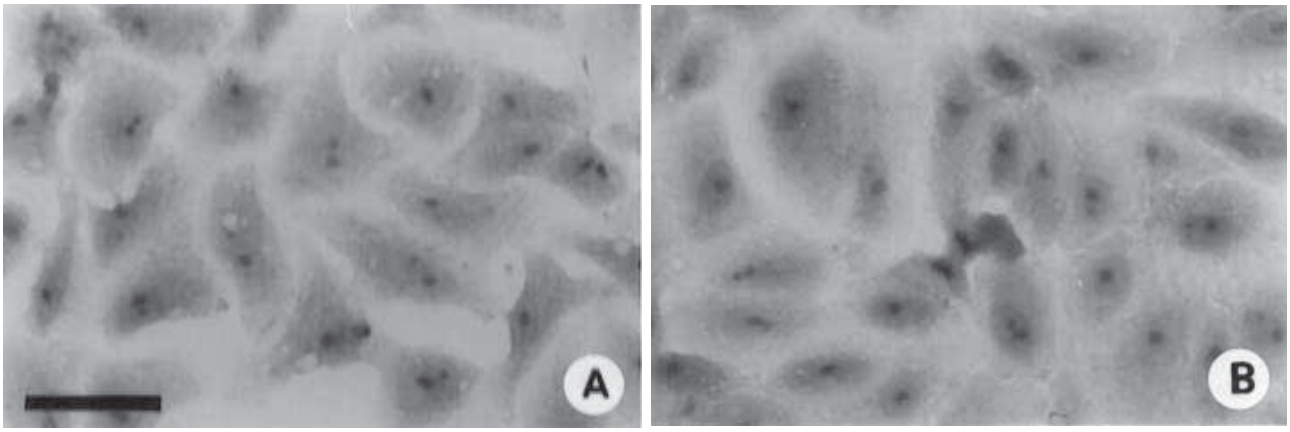

Figure 1. Light microscopy (crystal violet staining) of the indirect effect of a PFOC extract on the morphology of cultured Vero cells. **(A)** Control Vero cells, **(B)** PFOC extract-treated Vero cells. Note the flattened irregular morphology and 1-2 nucleoli per cell. Scale bar = 50 μm.

Immunocytochemical analysis

Staining with anti-actin monoclonal antibody markers indicated that in the negative controls the cells formed a semiconfluent monolayer, with thin actin filaments filling the cytoplasm. No stress fibers or other organized forms of actin filaments were seen (Fig. 2A,B). Cells cultured for 12 h under the weight of a glass coverslip showed more irregular contours along their borders. Actin was observed more clearly close to the cell periphery (Fig. 2C,D). In cells treated with silicone (a weight equivalent to that of PFOC – positive control), the morphology was irregular and fragmented because of disorganized actin filaments (Fig. 2E). Cells in direct contact with PFOC showed irregular morphology or fragmention, but with less actin, although there were small, actin-rich cell fragments. No other cells were observed apart from those in the transition zone (Fig. 2F,G).

Scanning electron microscopy

In scanning electron microscopy, the negative controls showed a semiconfluent cellular monolayer of flattened cells with some vesicles on the cell surface (Fig. 3A,B). Cells cultured for 12 hours under the weight of glass coverslip were irregular, with very thin edges and cytoplasmic retraction (Fig. 3C,D). Cells in direct contact with PFOC showed a markedly irregular morphology in the transition zone. The borders of the cells were retracted and there were cytoplasmic prolongations (Fig. 3E,F). Cell fragments were seen following direct contact with PFOC.

DISCUSSION

Recent studies on the effect of PFCLs on cell proliferation and viability have suggested that cellular injury may not be caused by direct toxicity, but rather by a mechanical or other indirect effect on cell metabolism. Other physiopathological mechanisms may also be involved [14].

In this study, we examined the possible mechanical and toxic effects of PFOC on cultured Vero cells. Some studies *in vitro* have used emulsified perfluorocarbons without consideration for their compressive effect on cells. Meller *et al*. [13] tested these liquids directly on cells to assess the damage caused by compression, but did not include a control that would produce "inert" non-toxic compression [14], a weight similar in size and area to the products tested on the cell layer. Such controls were included in our experiments.

The indirect cytotoxicity test showed that PFOC did not release substances harmful to cells. This finding agrees with the conclusions of others that alterations are contact-dependent and are observed after a short period of contact [19]. The areas showing cell death (i.e., no cells) were not significantly different among the treatment, an observation which further corroborated the lack of indirect cytotoxicity.

Marked morphological changes occurred in cells treated with PFOC, as shown by the immunocytochemical analysis for actin filaments. The cytoskeletal changes observed agreed with Meller *et al*. [13], who also reported toxicity to structural proteins (neurofilaments and tubulin) based on immunocytochemical analysis. Structural damage was also seen in scanning electron microscopy. The decrease in the number of cells was significant, with the changes caused by PFOC being more intense than those in the cells cultured on glass slides.

Although the cytotoxicity tests revealed that PFOC released no toxic substances into the culture medium, PFOC did produce cellular toxicity through direct contact with the cells. Similar findings were reported by Velikay *et al*. [20,21], who assessed the retinal effects of PFCL of a highly purified and a nonpurified formulation. The alterations observed included retinal necrosis in the PFCL transition zone (regardless of the formulation), which increased with the contact time, i.e. the changes were not simply a mechanical effect. These authors noted that the alterations caused by the unpurified formulation started earlier. Cellular injury was also dose-dependent, as described by Meller *et al*. [13], when different concentrations of emulsified perfluorocarbons were added to neuronal cell cultures. These alterations, which were restricted to the area in contact with PFOC, agree with the other studies reporting cellular changes only when the tissues were in contact with the test substance [2,5].

In conclusion, although a mechanical effect of PFOC may have altered the normal cell activity, our results also suggest the involvement of other mechanisms, as previously reported [14,19]. Such mechanisms may involve a direct toxic action on the cells.

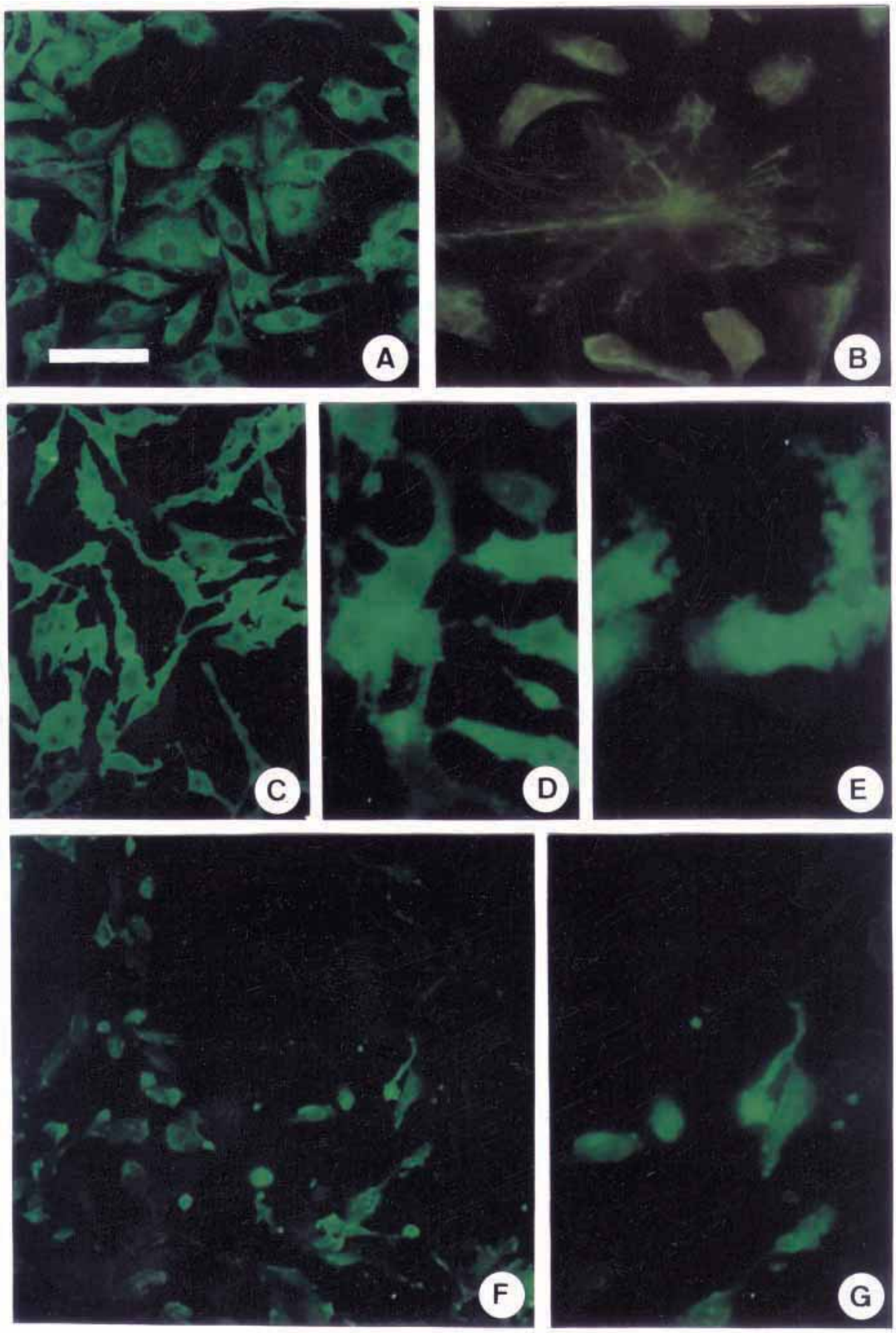

Figure 2. Immunocytochemical analysis of Vero cells cultured for 12 h and incubated with anti-actin monoclonal antibodies. **A**) and **B**), Negative controls (no chemical or mechanical action), **C**) and **D**), Cells cultured with glass coverslips (weight equivalent to that of PFOC), **E**) Cells cultured with silicone (weight equivalent to that of PFOC), **F**) and **G**), Cells under the compressive effect of PFOC – transition zone. Scale bar = 100 μm (B, D, E and G) or 50 μm (A, C, and F).

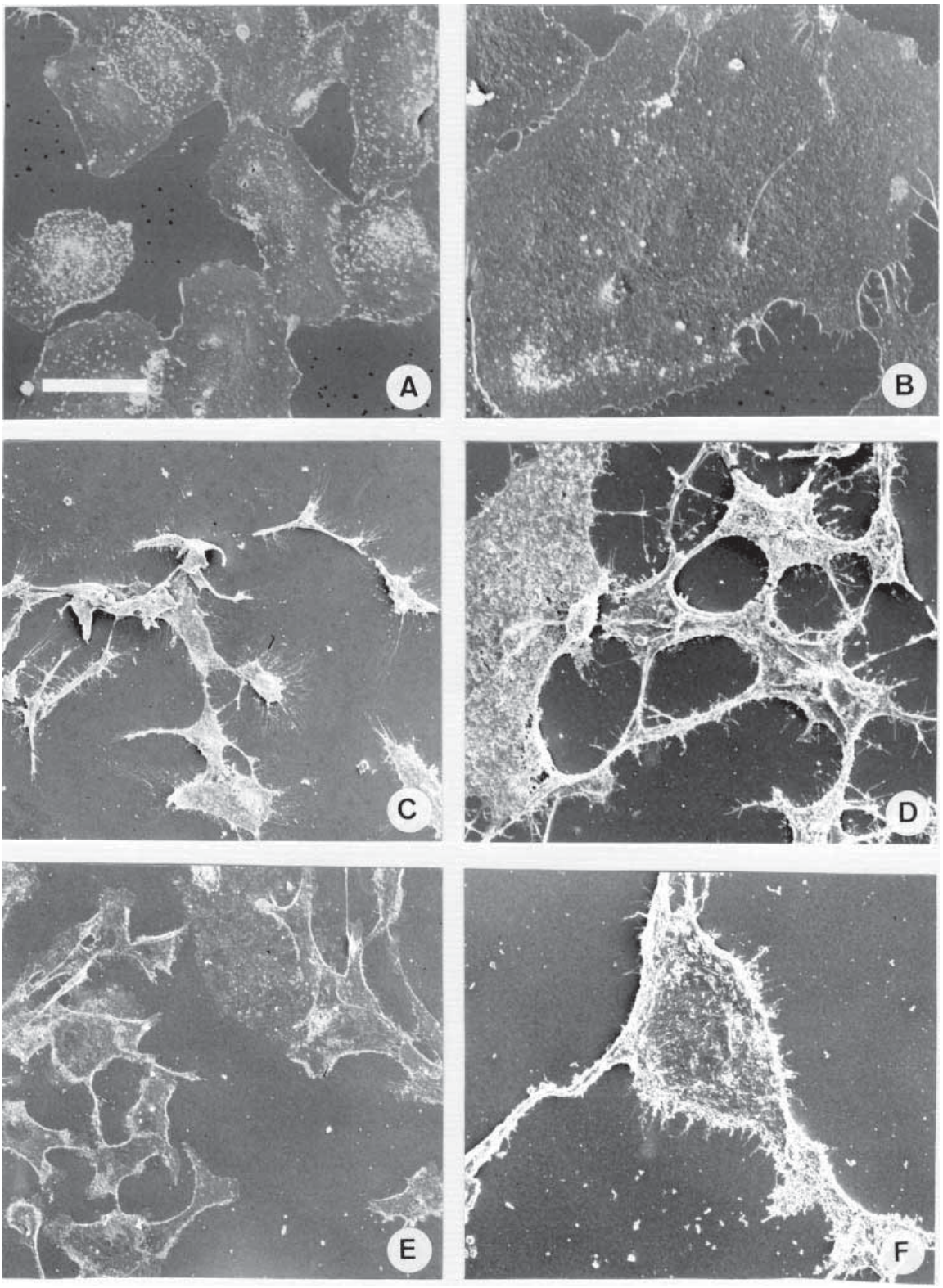

Figure 3. Scanning electronic microscopy of Vero cells. **A**) and **B**), Negative (no chemical or mechanical action), **C**) and **D**), Cells cultured with glass coverslips (weight equivalent to that of PFOC), **E**) and **F**), Cells under the compressive effect of PFOC. Scale bar = 70 µm (B, D and F) or 20 µm (A, C and E).

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