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Impacts of two perfluorinated compounds (PFOS and PFOA) on human hepatoma cells: Cytotoxicity but no genotoxicity?

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ABSTRACT

Perfluorinated compounds (PFCs) and particularly two of them, perfluoroctanoate (PFOA) and perfluorooctanesulfonate (PFOS), have been widely produced and used since 1950. They both persist in the environment and accumulate in wildlife and humans. The toxicity of PFOS and PFOA has been studied extensively in rodents with several adverse effects mainly a hepatocarcinogenic potential. Carcinogenic effects are not highlighted in humans' studies. In this study, we investigated the cytotoxic and genotoxic effects of PFOA and PFOS using human HepG2 cells after 1 or 24 h of exposure. The cytotoxic and genotoxic potential was evaluated by MTT assay, single cell gel electrophoresis (SCGE) assay and micronucleus assay respectively. We measured the intracellular generation of reactive oxygen species (ROS) using dichlorofluorescein diacetate to identify a potential mechanism of toxicity. We observed a cytotoxic effect of PFOA and PFOS after 24 h of exposure starting from a concentration of 200 μM (MTT: −14.6%) and 300 μM (MTT: −51.2%) respectively. We did not observe an increase of DNA damage with the comet assay or micronucleus with the micronucleus assay after exposure to the two PFCs. After 24 h of exposure, both PFOA and PFOS highlight a decrease of ROS generation (−5.9% to −23%). We did not find an effect after an hour of exposure. Our findings show that PFOA and PFOS exert a cytotoxic effect on the human cells line HepG2 but nor PFOA or PFOS could induce an increase of DNA damage (DNA strand breaks and micronucleus) or reactive oxygen species at the range concentration tested. Our results do not support that oxidative stress and DNA damage are relevant for potential adverse effects of PFOA and PFOS. These results tend to support epidemiological studies that do not show evidence of carcinogenicity.

Introduction

The perfluorinated compounds (PFCs) are characterized by a fully fluorinated hydrophobic linear carbon chain attached to various hydrophilic heads. PFCs do not occur naturally, they are man-made chemicals. They have been produced since the 1950s due to their unique properties such as anti-wetting or surfactant. Thus, they have been extensively used in industry and consumer products such as oil and water repellant, coating for cookware, carpets or textiles. Most physicochemical characteristics of PFCs are due to the extreme resistance of the carbon–fluorine (C–F) bond. Consequently this bond contributes to make PFCs resistant to many ways of degradation like heat, reaction with strong acids or bases, oxidizing agents or photolysis (Andersen et al., 2008). Several PFCs have been detected in nearly all environmental media and biota reflecting the wide-spread global pollution in all parts of the ecosystem (Giesy and Kannan, 2001; Martin et al., 2004). PFCs have also been detected in human blood and tissue samples from occupationally and non-occupationally exposed humans (Ericson et al., 2007; Holzer et al., 2008; Karrman et al., 2007; Volkel et al., 2008).

Among PFCs, perfluorooctane sulfonate (PFOS) and perfluorooctanoate acid (PFOA) are the most studied because they have been found to be the two predominant PFCs in environment, wildlife and human. Several human biomonitoring studies reveal a background exposure of the general population to PFOA and PFOS in many parts of the world (Kannan et al., 2004; Lau et al., 2007; Olsen et al., 2005).

The persistence of PFCs in the environment, their potential to accumulate in organisms and their occurrence in human tissues led to a rising concern about their possible adverse effects on human health. The toxicity of PFOS and PFOA has been studied extensively, mainly in rodents. Several reviews are available (Andersen

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et al., 2008; Kennedy et al., 2004; Lau et al., 2007). Hepatotoxicity, developmental toxicity, immunotoxicity, hormonal effects and a carcinogenic potency are the most worrying effects. In contrast, human studies are scare. Epidemiological and medical surveillance studies have been conducted on occupationally and non-occupationally exposed people. All occupationally exposed studies failed to find consistent effects on morbidity and mortality in humans (Emmett et al., 2006; Olsen et al., 2003, 2004, 2005). Recently, several non-occupationally exposed studies focuses on human developmental outcomes (Olsen et al., 2009) or thyroid disease (Melzer et al., 2010). The first case of serious environmental contamination in Germany, described by Wilhelm et al. (2008), highlighted a fourfold to eightfold increase of PFOA level in human plasma for people exposed to contaminated drinking water. An one-year follow-up study has demonstrated a slow decrease (10–20%) of PFOA-concentrations in blood plasma in relation to the use of activated charcoal filters before distribution of initially contaminated water (Holzer et al., 2009). A recent study contributed to elaborate a reference value for PFOA and PFOS in human plasma (Wilhelm et al., 2009). This study summarized three human biomonitoring studies with a non or exposed cohorts and proposed reference values of 10 μg/L for PFOA and 10, 15 and 25 μg/L for PFOS respectively for children, adult females and male adults.

The effects in rodents are due to the mode of action of PFOA and PFOS acting as peroxisomal proliferators (Andersen et al., 2008). Humans appear to be less sensitive or insensitive to carcinogenic potency effects of peroxisome proliferators. More recent studies (Hu and Hu, 2009; Yao and Zhong, 2005) used genotoxicity assays in human cells to evaluate the relationship between genotoxicity and oxidative stress.

The MTT assay is used routinely to evaluate both cytotoxicity, viability and vitality (growth of cells) of various cell lines (Mosmann, 1983; Scudiero et al., 1988). The comet assay is widely used for detecting genotoxic compounds (Hartmann et al., 2001). In both assays, a wide range of cell lines can be used, including HepG2 (Knasmuller et al., 1998, 2004). This cell line retains endogenous bioactivation capacity and thus contains several enzymes responsible for the activation of various xenobiotics (Diamond et al., 1980; Sassa et al., 1989). This line also retains many of the morphological characteristics of liver parenchymal cells. Thus, this cell line has been widely and successfully used for direct and indirect mutagens screening and for identifying antimutagens (Uhl et al., 2000; Valentin-Severin et al., 2003).

The aim of our study is to contribute to the evaluation of the cytotoxic and genotoxic effects of PFOA and PFOS using human HepG2 cells and the intracellular generation of reactive oxygen species in the same cell line.

Materials and methods

Materials

The HepG2 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) (HB-8065™). Dimethyl sulfoxide (DMSO), Dulbecco's Phosphate Buffered Saline (DPBS), ethidium bromide (EtBr), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein-diacetate (DCF-DA), dimethyl sulfoxide (DMSO), low melting point agarose, normal melting point agarose were purchased from Sigma–Aldrich (St Louis, USA).

All tissue culture reagents, i.e. Dulbecco's minimal essential medium (DMEM) containing Earl's salts and L-glutamine, heat-inactivated fetal bovine serum, penicillin, streptomycin and trypsin/EDTA solution were supplied by Sigma–Aldrich (St Louis, USA).

PFOA (CAS No. 335-67-1; purity ≥96%) and PFOS (CAS No. 1763-23-1) were supplied by Sigma–Aldrich.

Cell culture and treatment of cells

HepG2 cells were maintained in DMEM in a humidified incubator under 5% CO_2 at 37 °C. The DMEM was supplemented with 10% (v/v) fetal bovine serum, 10,000 IU of penicillin and 100 mg of streptomycin per milliliter, sodium pyruvate and HEPES. To maintain the cells cultures, DMEM is changed 3 times per week. Each week, sub confluents cultures are seeded in a new tissue flask of 25 or 75 cm^2 with a subcultivation ratio of 1:4–1:6.

PFOS and PFOA were dissolved in DMSO at various useful concentrations. In each assay, HepG2 cells were exposed to different concentrations of PFOA or PFOS by adding an appropriate volume of the stock solution of PFOA or PFOS in DMSO to the culture medium. DMSO concentration was carefully adjusted so that all working concentration of reagents (including controls) received exactly 2.5% volume percent (v/v) DMSO. To assess direct and indirect effects on the cells, we used two times of exposure: 1 h and 24 h. To conduct the experiments under standardized conditions, the culture time after seeding and the time of exposure was the same for each type of exposure time. For each assay, subculture was incubated during 48 h before exposed the cells to the toxicants. During this period, the cells recovered from the stress related to their division and proliferated in cell layer.

Viability assay

Our viability assay is based on a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The MTT is reduced by metabolically active cells in purple formazan. For the assay, we used a working solution at 5 mg/mL of PBS prepared and conserved at 4 °C in the dark. HepG2 cells were seeded in a 24-well culturing plates at a density of 2×10^5 cells/well in 1 mL of culture medium. After 48 h of incubation, the cells were treated in triplicate with DMSO (negative control) and with PFOA or PFOS in an indicated range of concentrations (5–800 μM). One well was not treated for control. The treated cells were cultured separately for 1 h or 24 h. The cells were treated with 500 μl of a MTT working solution and they were cultured for 2 h. The formazan is dissolved with 500 μl of DMSO and incubated for 10 min. Absorbance at 549 nm was read with a ThermoFischer Varioskan microplate reader. The experiment was repeated fivefold.

Morphological assay

HepG2 cells were seeded in 10 cm^2 round tissue flask containing one sterilized microscope slide at a density of 2×10^5 cells/tissue flask. The cells were cultured in 10 mL of culture medium for 48 h. The cells were treated with PFOA or PFOS in an indicated range of concentrations from 5 to 400 μM. One tissue flask was treated with DMSO 2.5% (v/v) and one was not treated for control. After separate culture during 1 or 24 h, the microscope slides were examined with a Leica microscope, and the observed changes described.

Single cell gel electrophoresis (SCGE) comet assay

SCGE assay was performed according to Tice et al. (2000) with slight modifications. The selection of PFOA and PFOS dosage was based on the results of vitality assays. Exposure time was 1 h or 24 h. In the 1 h SCGE assay, a 75 cm^2 confluents cells tissue flask was seeded in centrifugation tube. In the 24 h SCGE assay, HepG2 cells were seeded in a 25 cm^2 tissue flask and cultured for 24 h. In the two cases, the cells were exposed for 1 h or 24 h to PFOA and PFOS in a range of concentration respectively from 5 to 400 μM and from 5

to 300 μM. The negative control was made with 2.5% DMSO and the positive control with hydrogen peroxide for 1 h and benzo[*a*]pyrene for 24 h. The cells are collected, washed with DMEM and resuspended in DMEM at a concentration range between 2×10^5 and 2×10^6 cells/ml. The cell suspension (50 μl) was mixed with trypan blue solution and microscopically checked for membrane integrity. Only cell suspensions with viabilities above 90% were used for determination of DNA damage, to avoid artifacts resulting from cell death. Then 100 μl cell suspensions were immersed in 600 μl of 1% low melting point agarose, layered onto microscope slides precoated with 200 μl of 1% normal melting point agarose and spread with a coverslip. After solidification the slides were immersed in cold fresh lysing solution at pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100) for 1 h at least. Afterwards, the slides were placed for 20 min in a horizontal gel electrophoresis tank filled with cold electrophoretic buffer at pH 13 (1 mM Na_2EDTA and 300 mM NaOH) to allow DNA unwinding. Electrophoresis was performed in the same buffer for 20 min at 24 V (1 V/cm) and 300 mA. After electrophoresis, the slides were neutralized twice with 0.4 M Tris (pH 7.5). The slides were stained with 50 μl of ethydium bromide (20 μg/ml) just before analysis. Finally, the images were taken by fluorescence microscope (Olympus BX-40, Omachi, Japan) equipped with a 549 nm excitation filter and a 590 nm barrier filter. Hundred randomly selected cells (50 cells from each of the two replicate slides) were analyzed per sample with Komet 5 software (Kinetics Imaging). We retained 2 parameters of the comet: % TailDNA (percentage of DNA in the tail) and Olive Tail Moment (length between center of head and center of the tail multiplied by TailDNA). The experiment is repeated 5 times for each toxic and time of exposure.

Micronucleus assay (MN assay)

MN assay was performed according to Natarajan and Darroudi (1991) with slight modifications. HepG2 cells were seeded in 10 cm^2 round tissue flask containing one sterilized microscope slide at a density of 2×10^5 cells/tissue flask. The cells were cultured in 10 mL of culture medium for 48 h. For each PFC concentration and control, two independent cultures were prepared. The cells were treated for 24 h with PFOA or PFOS in an indicated range of concentrations from 5 to 400 μM. Mitomycin C and DMSO 2.5% (v/v) were used as positive and negative controls, respectively. Cytochalasin B, at a final concentration of 6 μg/ml, was added after treatment for 24 h. Cells were subjected to hypotonic treatment (5 mL KCl 0.075 M, 4 °C, 7 min). Cells were fixed 5 min with methanol/acetic acid (3:1 vol.). Finally, after drying, cells were stained with a Giemsa solution for 8 min. All slides were coded before scoring, which was carried out by the same person and microscope (Olympus BX-40, Omachi, Japan) at 1000× magnification, under oil immersion. The criterion for scoring MN was that described by Kirsch-Volders et al. (2000). For each slide, five hundred binucleated cells were scored and classified, according to the number of MN. For each experiment, two slides were scored for the induction of MN. Two experiments were realized and the values presented correspond to the pooled data from the two experiments (two thousands binucleated cells).

Measurement of intracellular reactive oxygen species (ROS)

The generation of ROS was detected using 2,7-dichlorofluorescein-diacetate (DCF-DA). DCF-DA acted as a fluorochrome to measure intracellular generation of ROS. HepG2 cells were seeded in a 24-well culture plates at a density of 2×10^5 cells/well in 1 mL of culture medium. After 48 h of incubation, the cells were treated in triplicate with PFOA or PFOS ranging from 5 to 400 μM. Three wells were not treated for negative control, three wells with DMSO 2.5% (v/v) and another three wells for positive control with 100 μM H_2O_2. They were incubated for 1 h or 24 h separately. After exposure, the wells were rinsed twice, and 500 μl of fresh DPBS with 10 μM DCF-DA was added. The cells were incubated for 45 min at 37 °C. The relative fluorescence intensity of the cell suspensions was measured by fluorescence microplate reader (ThermoFischer Varioskan, USA) with excitation wavelength 485 nm and emission wavelength 529 nm. To consider the mortality rate after treatment, the relative fluorescence intensity was weighted with the viability results.

Statistical analysis

The experiment was used as the experimental unit. A result was considered statistically significant at $p < 0.05$. The exact p-value was not described because of small samples. The statistical analysis was realized with SPSS v17.0. ANOVA or Kruskall–Wallis with adapted post hoc test was used. For the MN assay, data for the binucleated cells with MN were compared for each treatment using Pearson's Chi-Square test and Fisher's exact test.

Results

HepG2 viability after treatment by PFOA or PFOS

The results are presented in Fig. 1 (PFOS) and Fig. 2 (PFOA). After 1 h of exposure, we did not observe statistically significant variation, in comparison with the control, neither for PFOA ($p = 0.22$) nor for PFOS ($p = 0.15$). The range of variations was −2.1% (PFOS

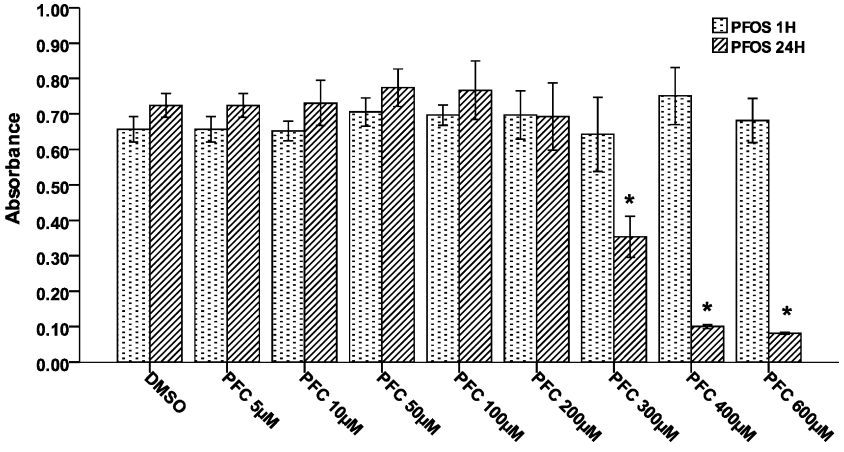

Fig. 1. Results of viability assay for PFOS after 1 h or 24 h of exposure. Means ± 1 SD. *Statistically different at $p = 5\%$ from DMSO 2.5% ($n = 5$).

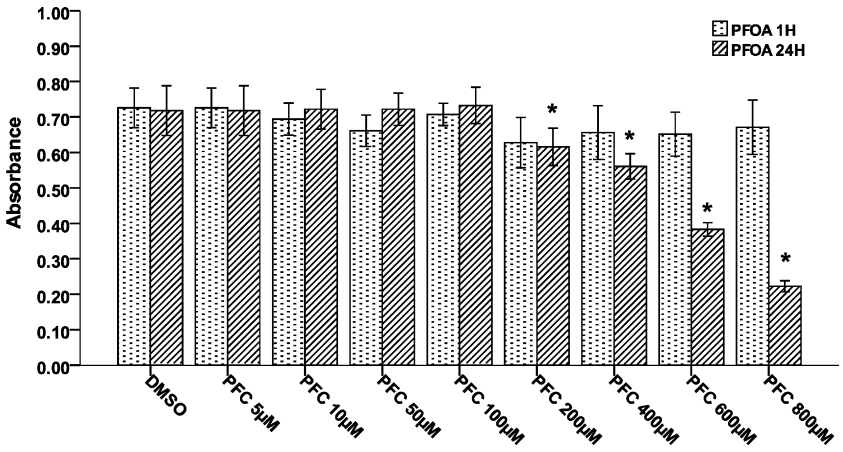

Fig. 2. Results of viability assay for PFOA after 1 h or 24 h of exposure. Means ± 1 SD. *Statistically different at $p = 5\%$ from DMSO 2.5% ($n = 5$).

300 μM) to 14.3% (PFOS 400 μM) for PFOS and −13.5% (PFOA 200 μM) to −2.5% (PFOA 100 μM) for PFOA. For 8 PFOS concentrations tested, only 2 have a negative change relative to control (PFOS 10 and 300 μM). For 8 PFOA concentrations tested, all have a negative change relative to control. We did not observe dose/response relations for PFOA and PFOS after 1 h of exposure. After 24 h of exposure, we observed statistically significant variation, as compared with the control, for both PFOA ($p < 0.05$) and PFOS ($p < 0.05$). For PFOS, we observed statistically significant variation ($p < 0.05$) at PFOS 300 μM (−51.2%), 400 μM (−86.2%) and 600 μM (88.8%). Cytotoxic effect appeared at 300 μM with dose–response relation effect and without visible effect at lower concentrations. For PFOA, we observed statistically significant variation at PFOA 200 μM (−14.2%), 400 μM (−21.9%), 600 μM (46.7%) and 800 μM (−69.0%). Cytotoxic effect appeared at 200 μM with dose-–response relation effect and without visible effect at lower concentrations. For the further assay, we used concentrations from 5 to 300 μM for PFOS and from 5 to 400 μM for PFOA.

Morphological changes after treatment by PFOA or PFOS

After 48 hours incubation, the cells begin to form a monolayer. The cells were elongated and were able to produce an extracellular matrix between cells. After 1 h or 24 h of exposure to DMSO 2.5%, the macroscopic and microscopic appearance of cells was similar to the cells in the culture medium control. After 1 h of exposure to PFCs, we could not observe morphological differences. After 24 h of exposure and from a concentration of 200 μM for PFOA and 300 μM for PFOS, we observed intracellular vacuoles in several cells, a disappearance of the extracellular matrix and a substantial number of cells suspended in culture medium. Among the adherent cells, small numbers were rounded. Beyond 600 μM of PFOA and 400 μM of PFOS, we noted a significant increase in suspended cells and cell debris.

DNA strand breaks induced by PFOA or PFOS

The results of SCGE assay are presented in Tables 1 and 2 for PFOA and PFOS respectively. The results of the Trypan blue viability assay are not presented. The results were similar with MTT assay. The viability was above 80% for all compounds and exposures times excepting for PFOS 300 μM at 24 h.

The results of positive control are not presented here. There were statistically different at $p = 0.05\%$ from the negative control for hydrogen peroxide (1 h, mean of 2-fold increase) and benzo[*a*]pyrene (24 h, mean of 3-fold increase). After 1 h of exposure, we did not observe statistically significant variation, in comparison with the control, for the two parameters: %TailDNA (PFOA, $p = 0.71$ and PFOS, $p = 0.67$) and Olive Tail Moment (PFOA, $p = 0.99$ and PFOS, $p = 0.56$). For PFOA, we observed variations from 3.3% (PFOA 400 μM) to 21.3% (PFOA 10 μM) for % TailDNA and from −4.3% (PFOA 200 μM) to 0.9% (PFOA 5 and 10 μM). For PFOS, we observed variations from 15.6% (PFOS 5 μM) to 46.1% (PFOS 100 μM) for % TailDNA and from 28.1% (PFOS 200 μM) to 172% (PFOS 100 μM) for Olive Tail Moment. We did not observe dose/response relations for PFOA and PFOS after 1 h of exposure. Nevertheless, the results are above the negative control for PFOS and PFOA.

After 24 h of exposure, we observed statistically significant variation, comparatively to the control, for the two parameters: %TailDNA (PFOA, $p < 0.05$ and PFOS, $p = 0.16$) and Olive Tail Moment (PFOA, $p < 0.05$ and PFOS, $p = 0.67$). PFOA at 10 μM and 200 μM were statistically different at $p = 0.05$ with Dunnett's post hoc test. For PFOA, we observed variations from 18.3% (PFOA 50 μM) to 64.8% (PFOA 200 μM) for %TailDNA and from 19.3% (PFOA 50 μM) to 80.4% (PFOA 200 μM) for Olive Tail Moment. For PFOS, we observed variations from −19.7% (PFOS 5 μM) to 24.3% (PFOS 200 μM) for %TailDNA and from −21.3% (PFOS 5 μM) to 55.3% (PFOS 200 μM). We did not observe dose/response relations for PFOA and PFOS

Table 1
Results of SCGE assay for PFOA (mean ± 1SD, $n = 5$).

Toxic	%TailDNA		Olive tail moment	
	1 h	24 h	1 h	24 h
DMSO 2.5%	2.45 ± 0.77	2.45 ± 0.15	0.25 ± 0.12	0.30 ± 0.03
PFOA 5 μM	2.77 ± 0.67	3.53 ± 0.37	0.29 ± 0.15	0.48 ± 0.10
PFOA 10 μM	3.04 ± 0.79	3.88 ± 1.15[a]	0.30 ± 0.15	0.52 ± 0.22[a]
PFOA 50 μM	2.90 ± 0.29	2.90 ± 0.72	0.27 ± 0.11	0.36 ± 0.08
PFOA 100 μM	2.60 ± 0.56	3.02 ± 0.66	0.26 ± 0.12	0.37 ± 0.09
PFOA 200 μM	2.78 ± 0.61	4.03 ± 0.76[a]	0.27 ± 0.14	0.55 ± 0.14[a]
PFOA 400 μM	2.61 ± 0.55	3.18 ± 0.49	0.26 ± 0.09	0.41 ± 0.05

[a] Statistically different at $p = 5\%$ from DMSO 2.5%.

Table 2
Results of SCGE assay for PFOS (mean ± 1SD, $n = 5$). Statistically different at $p = 5\%$ from DMSO 2.5%.

Toxic	Tail-DNA		Olive tail moment	
	1 h	24 h	1 h	24 h
DMSO 2.5%	3.10 (4.27)	3 (3.69)	0.35 (0.50)	0.30 (0.40)
PFOS 5 μM	3.58 (7.40)	2.40 (3.28)	0.62 (3.97)	0.23 (0.31)
PFOS 10 μM	3.99 (9.60)	3.13 (3.79)	0.69 (3.72)	0.31 (0.38)
PFOS 50 μM	3.87 (5.08)	2.71 (3.82)	0.48 (0.69)	0.28 (0.41)
PFOS 100 μM	4.52 (7.85)	3.02 (4.25)	0.96 (7.72)	0.28 (0.44)
PFOS 200 μM	3.7 (5.03)	3.72 (7.99)	0.45 (0.70)	0.46 (2.07)
PFOS 300 μM	4.08 (5.73)	–	0.50 (1.46)	–

Table 3
MN frequency ‰ in HepG2 cells treated with PFOA or PFOS for 24 h (mean ± 1SD; $n = 5$). *Statistically different at $p = 5\%$ from DMSO 2.5%.

Toxic	PFOA	PFOS
DMSO 2.5%	25.7 (±2.1)	23.3 (±3.5)
PFC 5 μM	21.7 (±1.2)	23.0 (±1.7)
PFC 10 μM	23.3 (±3.1)	23.0 (±2.0)
PFC 50 μM	24.3 (±3.1)	21.7 (±2.5)
PFC 100 μM	24.7 (±2.1)	24.3 (±1.5)
PFC 200 μM	25.7 (±2.5)	23.3 (±1.5)
PFC 300 μM		24.3 (±2.1)
PFC 400 μM	25.3 (±2.1)	

after twenty-four hours of exposure. Nevertheless, the results are consistently above the negative control for PFOA and PFOS.

Micronucleus assay

The micronucleus assay evaluated the induced frequency of micronucleus in binucleated HepG2 cells. The results are presented in Table 3. Positive controls induced a 3.8-fold increase with a significant statistical difference ($p < 0.05$). Neither PFOS nor PFOA induced an increase of micronucleus frequency ($p = 0.995$ and $p = 0.952$ respectively) in comparison with DMSO 2.5%.

Oxidative stress: generation of reactive oxygen species

The results are presented in Figs. 3 and 4 for PFOS and PFOA respectively. For one hour of exposure, we did not highlight any statistical difference between DMSO 2.5% and PFOA ($p = 0.83$) or PFOS ($p = 0.82$). For PFOA, the variations are included between −13.2% (PFOA 50 μM) and −2.1% (PFOA 10 μM). For PFOS, the variations are located between −5.7% (PFOS 300 μM) and 6.1% (PFOS 100 μM). For 24 h of exposure, we did not highlight any statistical difference between DMSO 2.5% and PFOA ($p = 0.81$) or PFOS ($p = 0.26$). For PFOA, the variations are included between −5.9% (PFOA 5 μM) and −20.2% (PFOA 100 μM). The PFOA's results were always below the negative control (DMSO) with no dose/response relation. For PFOS, the variations are located between −5.9% (PFOS 5 μM) and −23.0% (PFOS 200 μM). For PFOS, we observed a peak of ROS generation at 300 μM for PFOS (40.6%). The PFOS's results highlighted a negative dose/response relation with an "aberrant" result at PFOS 300 μM.

Discussion

PFCs, including PFOA and PFOS, are ubiquitous pollutants. Their occurrence in human's tissues has led to study their impact on ecosystems and living beings (Kannan et al., 2004; Lau et al., 2007; Olsen et al., 2005). Early toxicity studies showed a significant effect in animals including a carcinogenic effect in rodents (Andersen et al., 2008; Kennedy et al., 2004; Lau et al., 2007). However, epidemiological studies have not revealed significant causal link between exposure to PFCs and diseases (Emmett et al., 2006; Olsen et al., 2003; Olsen et al., 2004; Olsen et al., 2005). In the present study, we investigated the toxic effects of PFOS and PFOA on cells viability, DNA integrity and ROS generation in HepG2 human cells by using four end-points: mitochondrial survival, primary DNA lesions (single- and double-strand breaks and alkalabile sites), micronucleus generation and ROS generation.

Both viability and cellular morphology assays showed a cytotoxic effect of both PFOS and PFOA. This cytotoxic effect appeared only after twenty-four hours of exposure. One hour exposure failed to demonstrate a cytotoxic effect for all tested concentrations. The cytotoxic effect after 24 h appeared at 200 μM or 300 μM for PFOA or PFOS respectively with a dose–response effect. In the literature, other studies suggest a dose effect impact of cell viability after 24 h exposure (Hu and Hu, 2009; Shabalina et al., 1999). Hu and Hu (2009) found a cytotoxic effect at 150 μM of PFOS after 24 h

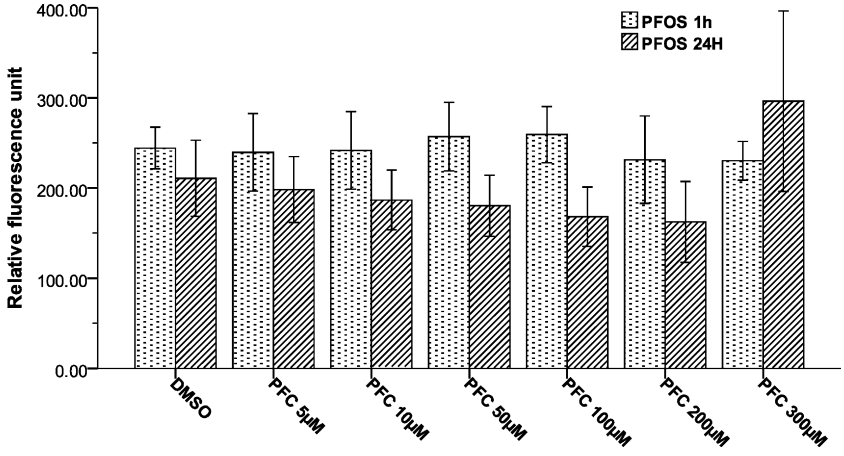

Fig. 3. Relative fluorescence of ROS generation after 1 h or 24 h of exposure to PFOS (mean ± 1SD). *Statistically different at $p = 5\%$ from DMSO 2.5% ($n = 5$).

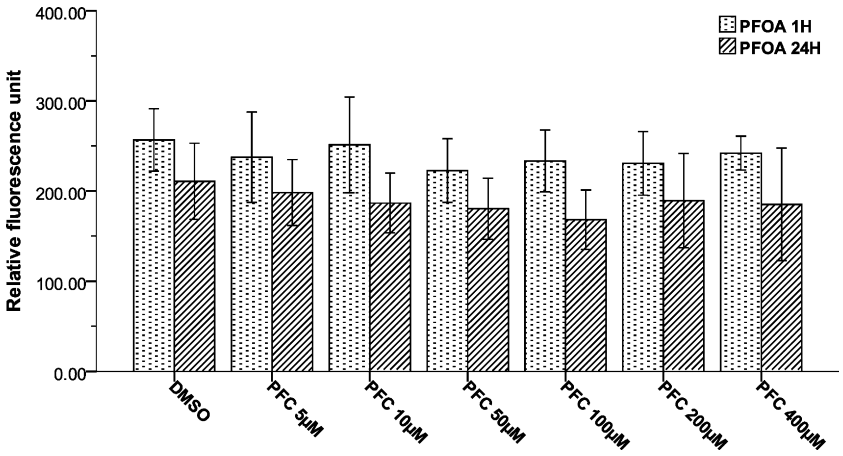

Fig. 4. Relative fluorescence of ROS generation after 1 h or 24 h of exposure to PFOA (mean ± 1SD). *Statistically different at $p = 5\%$ from DMSO 2.5% ($n = 5$).

of exposure. Shabalina et al. (1999) highlighted a cytotoxic effect at 200 μM of PFOA after 24 h of exposure. These results are close to our findings. The modifications of cells conformation observed may be considered like markers of cytotoxicity (appearance of vacuoles, loss of adhesion or presence of cellular remains). These cytotoxic effects could be related to the physico-chemical properties of PFCs, in particular their action of surfactant (Quinete et al., 2010). Surfactants demonstrate effective activities, such as detergency, emulsification, dispersion, and solubilization. Detergent's capacity of PFOA on human cells has been observed by Levitt and Liss (1986). The disappearance of extracellular matrix between cells and the rounding of cells could be explained by these activities.

PFOA and PFOS are carcinogenic in rodents but this effect has never been found by epidemiological studies in humans. Therefore we studied the mutagenic potential of these two compounds on human cells. We chose HepG2 cells, cells derived from human hepatocellular cancer, routinely used for testing genotoxicity and exhibiting a significant metabolic activity. In our study, we tested two times of exposure to evaluate direct and non-direct mutagenic effects. Our results show that PFOA (1 h) and PFOS (1 h, 24 h) did not cause significant increase of DNA damage in the comet assay. PFOA (24 h) at 10 and 200 μM showed a significant difference from negative control but not at 5, 50, 100 and 400 μM. These differences can be related to physiological changes during the procedure. Although the results are consistently higher than the negative control, we do not observe a dose–response relationship for both toxics. In the micronucleus assay, our findings have shown that PFOA and PFOS are not able to induce significant increase in the frequency of MN in binucleated cells. To investigate if PFOA and PFOS are able to induce excessive reactive oxygen species (ROS) production (oxidative stress), we measured ROS generation with DCF-DA. DCF-DA is cell-permeable and is almost only oxidized inside the cells to form fluorescent dichlorofluorescein in presence of ROS. Our results did not highlight an increase of ROS generation after PFOA or PFOS exposure. Our data do not support the notion that oxidative stress and DNA damage (strand break or micronucleus) are relevant for potential adverse effects of PFCs in humans.

A previous study (Yao and Zhong, 2005) reported that HepG2 cells exposed to PFOA (for 1 h) had a concentration-dependent increase in the level of strand breaks detected by the alkaline comet assay and of micronucleus detected by the micronucleus assay. Although the incubation time is different between this these two experiments, the comet-assay conditions do not differ to an extent that could explain the discrepancy. The same cells line and comparable range of concentrations were used. A recent study (Eriksen et al., 2010) did not show any variations of DNA damage with the comet assay. This study used a similar incubation time (24 h) and range of concentrations. In the literature, 4 studies measured ROS generation (Eriksen et al., 2010; Hu and Hu, 2009; Panaretakis et al., 2001; Yao and Zhong, 2005) mainly with DCF-DA assay on HepG2 cells. Our results are not in accordance with these studies. While Yao and Zhong (2005) showed a 4-fold increase of ROS generation after 3 h of exposure to PFOA; Eriksen et al. (2010) highlighted a modest increase (1.2 for PFOS and 1.5 for PFOA) with no concentration-dependence. Nevertheless, the procedure of measure of ROS generation is slightly different between those studies. This variation of assay conditions could explain the discrepancies. One possible hypothesis to explain the difference with our study could be the difference of cell state when adding the fluorescent marker. In our study, we tried to avoid the addition of a cellular stress, whereas other studies re-suspend the cells after trypsinization and pelleting. Trypsination and pelleting are known factors that induce a cellular stress. Two studies (Eriksen et al., 2010; Yao and Zhong, 2005) measured the formation of formamidopyrimidine-DNA-glycosylase (FPG)-sensitive sites in DNA with a modified comet assay. The two studies showed contradictory results. FP-sensitive sites represent some DNA damage linked with ROS. The invoked mechanism for genotoxicity and the generation of reactive oxygen species is the peroxisome proliferators' effect (related to PPARα-activation) of PFOA and PFOS. Nevertheless, the literature has revealed very different species sensitivities to this effect. Rodents are particularly susceptible while humans or nonhuman primates do not seem sensitive or only very little sensitive.

In this study, only single exposures of PFOA and PFOS were investigated. People are exposed to mixtures of chemicals. These mixtures can induce potentiation or interactions of the different effects of a toxic compound alone.

In summary, our findings show that PFOA and PFOS have a cytotoxic effect on human cells line HepG2 only at high concentrations and a long time of exposure. Nor PFOA or PFOS could induce an increase of DNA damage or micronucleus on HepG2 at the range of concentration tested. We did not highlight a ROS generation. Our results do not support that oxidative stress (ROS generation) and DNA damage (comet and micronucleus assay) are relevant for potential adverse effects of PFOA or PFOS. Our findings are consistent with the recent epidemiological study demonstrating an association between PFOA or FPOS and liver pathologies (Eriksen et al., 2009). However, the contradictory results of genotoxicity with the same cell line in the literature and in light of recent studies showing endocrine disruption potency (White et al., 2011) independently of peroxisome proliferation way, it appears necessary to test the impact of these perfluorinated compounds on other human cell lines.

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