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This is an **author produced version** of a paper published in:

Food and Chemical Toxicology 121 (2018): 351-359

DOI: <https://doi.org/10.1016/j.fct.2018.09.020>

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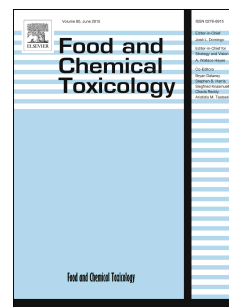
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Accepted Manuscript

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PII: S0278-6915(18)30664-1

DOI: [10.1016/j.fct.2018.09.020](https://doi.org/10.1016/j.fct.2018.09.020)

Reference: FCT 10046

To appear in: *Food and Chemical Toxicology*

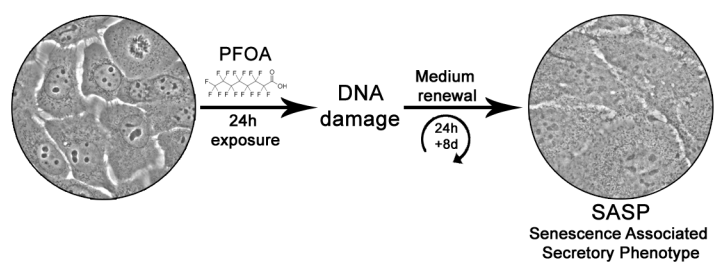
Received Date: 7 July 2018

Revised Date: 6 September 2018

Accepted Date: 11 September 2018

Please cite this article as: Peropadre, A., Freire, Paloma.Ferná., Hazen, Marí.José., A moderate exposure to perfluorooctanoic acid causes persistent DNA damage and senescence in human epidermal HaCaT keratinocytes, *Food and Chemical Toxicology* (2018), doi: 10.1016/j.fct.2018.09.020.

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A moderate exposure to perfluorooctanoic acid causes persistent DNA damage and senescence in human epidermal HaCaT keratinocytes.

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Abstract

Perfluorooctanoic acid has been used widespread, during the last decades, in a number of consumer and industrial products. Although this compound has been subjected to extensive epidemiological and toxicological studies, limited data are available concerning its potential dermal toxicity in mammalian cells. In this study, we used a two-stage approach with relevant cytotoxicity endpoints including cell viability and proliferation, oxidative stress, DNA damage and cell senescence to assess the immediate and the long-lasting or delayed cytotoxicity caused by the compound in HaCaT keratinocytes.

Our results suggest that a single exposure to perfluorooctanoic acid causes concentration-dependent changes in cell proliferation that were not restored during a 48 h recovery period. Furthermore, we demonstrate that a moderate treatment with this perfluorochemical causes persistent DNA damage, which ultimately leads to development of the senescence-associated secretory phenotype in HaCaT cells. This paper provides unprecedented data and insights regarding the cytotoxic effects of perfluorooctanoic acid in human cells that could be of special relevance for use in comparative *in vitro-in vivo* studies. Moreover, our findings highlight the importance of considering both the immediate and long-lasting or delayed cytotoxic responses caused by chemical exposure, to ensure the accurate identification of toxicity in cell-based systems.

Key words : Perfluorooctanoic acid, HaCaT cells, cytotoxicity, DNA damage, senescence.

Funding source

This work was supported by the Spanish Ministry of Economy and Competitiveness [grant number CTM2012-31344].

1. Introduction

Perfluorooctanoic acid (PFOA) is a long-chain perfluorinated chemical with unique lipid- and water-repellent characteristics that has been extensively used during the last decades in a number of consumer and industrial products, such as Gore-Tex and Teflon (Prevedouros et al. 2006). PFOA is extremely stable and persistent in the environment and has been detected worldwide in human serum samples from the general population (Lindstrom et al., 2011), thus raising environmental and public health concerns. The production and use of this perfluorinated compound has been phased out by the major manufacturers through a stewardship agreement with the U.S. Environmental Protection Agency (US EPA), designed to eliminate emissions and product content by 2015 (US EPA, 2006).

A number of studies, conducted mainly in rodents, have associated PFOA exposure with adverse hepatic effects and impairment of reproductive, immunological and endocrine functions (Lau et al., 2007; Post et al., 2012; White et al., 2011). PFOA has also been reported to induce pancreatic, liver, testicular and mammary tumors (Biegel et al. 2001; Butenhoff et al. 2012), mediated at least in part by peroxisome proliferator-activated receptor alpha (PPAR α) signalling. The relevance of these animal data for humans is still under debate because of interspecies variations in the adverse effects caused by PPAR α activation (Guyton et al., 2009; Rosen et al. 2009) and the marked differences in serum elimination half-lives of PFOA, ranging from days in rodents to 3.5 years in humans (Olsen et al. 2007). However, although available epidemiological studies have failed to provide conclusive evidence of a causal relationship between exposure to this perfluorinated compound and adverse health effects (Chang et al., 2014), PFOA has been considered under the European REACH legislation as a “Substance of Very High Concern” (ECHA, 2014) and classified by the International Agency for Research on Cancer (IARC) as “possibly carcinogenic to humans” (Group 2B) (IARC, 2016). These controversial results suggest the need for additional studies

to fully understand the mechanisms underlying the cytotoxicity and potential carcinogenicity of PFOA in humans.

Although animal experimentation still remains necessary, it is now widely accepted that cellular systems can provide mechanistic and more predictive data for human health risk assessment than conventional animal models (Huang et al., 2016). Moreover, PFOA is metabolically inert in mammals and therefore a particularly suitable compound for *in vitro* toxicity analysis. In this context, previous studies have reported that this perfluorinated compound, at a concentration range comparable to that used in this study, produces moderate cytotoxic effects in non-human mammalian cell lines (Coperchini et al., 2015; Fernández Freire et al., 2008; Mulkiewicz et al., 2007). On the other hand, while some results have clearly demonstrated that PFOA induces oxidative stress and apoptosis in both tumoral (HepG2) and normal (L-02) human liver cell lines (Hu and Hu, 2009; Huang et al., 2013; Panaretakis et al., 2001), other reports do not seem to support these findings (Buhrke et al., 2013; Eriksen et al., 2010; Florentin et al., 2011). The data from cell-based assays are directly influenced by the experimental design and the methodological approach adopted in the study. In most cases, cell cultures are exposed to the test substance during a single incubation period and cytotoxicity is immediately determined. These short-term experiments which are convenient as part of a tiered approach to chemicals evaluation, can often underestimate or even fail to detect delayed cell injury highly predictive for human toxicity (Dierickx, 2003).

The present study was particularly focused on evaluating the cytotoxic effects caused by PFOA exposure in epidermal HaCaT keratinocytes, recognized as suitable candidates for assessing human skin irritancy and toxicity under culture conditions (van de Sandt et al., 1999). Although absorption through the skin may be a significant route of exposure to this chemical, not only in occupationally exposed adults but also in the general population (Franko et al., 2012), limited data at the cellular level are available on the potential dermal toxicity of PFOA. Our results, obtained using a two-stage

approach and different endpoints to ensure the accurate identification of cytotoxicity, will provide further insight into the the short-and long-lasting adverse effects of PFOA in human cells that could be of special relevance for use in comparative *in vitro-in vivo* studies.

2. Materials and methods

2.1. Cell culture and PFOA treatments

HaCaT cells, obtained from Cell Lines Service (CLS, Germany), were routinely grown at 37° C in a 5% CO₂ humidified atmosphere, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (all from Lonza, Switzerland). In all experimental procedures, early-passage cells were used to avoid undesirable replicative senescence. Exponentially growing cells were seeded in different cell culture surfaces (Falcon, Becton Dickinson, USA), at a density of 10⁵ cells/mL for 24-h experiments and 5x 10⁴ cells/mL for longer experiments. After an overnight incubation, cells were treated with a new medium containing PFOA concentrations ranging from 10 to 500 µM (4-200 µg/mL), which were selected on the basis of our previous results (Fernández Freire et al., 2008). At given times during the exposure period (24 and 72 h), both treated cells and untreated control cells were gently washed with phosphate buffered saline (PBS) and processed according to the different analyses.

In recovery experiments, HaCaT cells pretreated for 24 h with the compound were washed and then incubated in complete drug-free medium for an additional 48 h (denoted 24+48 h). Besides, some supplementary assays were performed following a post-exposure period of 8 days (denoted 24+8 d). In these cases, cells initially seeded in 75 cm² flasks (5x 10⁴ cells/mL) were treated with PFOA for 24 h and then incubated in drug-free medium for 48 h. Afterwards, the cells were replated at subconfluent

density in different culture surfaces and maintained in complete culture medium for further 6 days (medium was refreshed by changing half of the volume after 3 days).

Stock solutions of 10^{-1} M PFOA (CAS number 335-67-1, Sigma, USA) were prepared in dimethylsulphoxide (DMSO) and maintained in darkness at room temperature. The working solutions were prepared before use in DMEM with 2% FCS and sterilized by filtration through a 0.22 μ m Millipore® filter. DMSO concentrations in medium did not exceed 0.1% including the control groups.

2.2. Cytotoxicity assays

Cytotoxic effects caused by continuous exposure to PFOA were evaluated by three quantitative spectrophotometric methods on HaCaT cells grown in 24-well plates and treated for 24 or 72 h with increasing PFOA concentrations. Cell growth and/or cell detachment was estimated by quantifying total protein content (TPC) according to the Bradford method (1976) using Coomassie Brilliant Blue G-250 reagent* (BioRad, Germany) and bovine serum albumin (Sigma) as standard. MTT assay, that involves the reduction of the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) by dehydrogenases of viable cells to purple formazan, was performed according to the method of Mosmann (1983). Neutral red uptake (NRU) into the lysosomes of viable cells was evaluated as described by Borenfreund and Puerner (1985). The absorbance values for the different cytotoxicity assays were recorded at appropriate wavelengths (TPC, 595 nm; MTT, 570 nm; NRU, 542 nm), using a Synergy• HT microplate reader (Biotek, USA).

In parallel, the mitotic index (MI) was determined in cells growing on glass coverslips into 12-well culture plates and treated with PFOA under the same experimental conditions. The cells were then fixed with cold methanol and stained 1 min with 5 μ g/mL Hoechst 33258 (Riedel de Haen, Germany). Three thousand cells were counted

in blind per experimental point, and the MI was calculated as the ratio between the number of cells in mitosis and the total number of cells.

To evaluate the potential reversibility of the cytotoxic effects, PFOA-treated cells were allowed to recover in fresh culture medium for 48 h and reassessed for TPC, MTT reduction, NRU and mitotic activity, as described previously.

2.3. Immunofluorescence staining

To evaluate mitotic spindle and chromosome organization, HaCaT cells grown on glass coverslips into 12-well culture plates were treated with 50 μ M PFOA for 24, 24+48 or 72 h and fixed in methanol at -20° C for 6 min. The cells were immunostained with an anti- α -tubulin antibody (Sigma,USA) and nuclei counterstained with with 5 μ g/mL Hoechst 33258, as described previously (Peropadre et al., 2011). The possible presence of disturbed spindles and/or chromosome congression failures was determined by examining at least 300 PFOA-treated mitotic cells in each experiment.

The production of the proinflammatory cytokine interleukin-6 (IL-6) was assessed by immunofluorescence in HaCaT cells grown on glass coverslips into 12-well culture plates and treated with 50 μ M PFOA (24 + 8 d). After fixation for 10 min with 4% paraformaldehyde in PBS, cells were incubated overnight at 4° C with anti-IL-6 antibody from R&D systems (Minneapolis, MN, USA), according to the manufacturer recommendation. Following extensive washing in PBS, coverslips were incubated for 45 min at 37° C with a secondary Alexa 488 antibody (Invitrogen,UK), counterstained for 1 min with 5 μ g/mL Hoechst 33258 and mounted in ProLong Gold antifading reagent (Invitrogen, UK). The percentage of IL-6-positive cells was determined by counting, under fluorescence microscopy, a total of 2500 cells per sample in randomly selected fields.

2.4. Cell cycle analysis

Cells were seeded in 75 cm² culture flasks and treated with 50 µM PFOA (24+8 d) for flow cytometry analysis of DNA content. Afterwards, both floating and adherent cells collected by trypsinization were processed as described elsewhere (Peropadre et al., 2015). Measurements were carried out in a Coulter Epics XL-MCL (Beckman Coulter, USA) cytometer equipped with an argon laser (488 nm) and the appropriate Photomultiplier to recover 620 nm light. Data were analyzed using the Expo 32 ADC software (Beckman Coulter).

2.5. Evaluation of genotoxic damage

The possible induction of DNA double-strand breaks (DSBs) was examined by indirect immunofluorescence against the phosphorylated form of the variant histone H2AX (γ -H2AX). HaCaT cells grown on glass coverslips into 12-well culture plates and treated with 50 µM PFOA were processed, as previously indicated (Pérez Martín et al., 2010), either immediately after a 24 h exposure to the compound or following a recovery period of 8 days in drug-free medium (24+8 d). A total of 1000 randomly chosen interphases were scored per slide, and divided into cells with and without DSBs depending on positive label to nuclear γ -H2AX foci. Cells considered positive for γ -H2AX demonstrated a minimum of 10 discrete foci of brightness.

In parallel, immunocytochemical detection of 8-OHdG was carried out essentially as described by Yarborough et al. (1996) with minor modifications. Cells were fixed with formaldehyde-PBS 1:10 (v/v) at 4°C for 20 min and treated with 100 µg/mL RNase A (Sigma) for 1 h at 37°C. Following DNA denaturation for 5 min at 4°C, samples were processed as previously described (Pérez Martín et al., 2010). The signal intensity of the 8-OHdG immunostaining of at least 100 randomly selected cells per experimental condition was measured using the Image J 1.41 software (National Institutes of Health, USA). The formula for quantification was as follows: 8-OHdG index = [(X-threshold) ×

area (μ^2)/total cell number, where X is the staining density indicated by a number between 0 and 256 on a grayscale.

2.6. Senescence-associated- β -galactosidase activity

HaCaT cells treated with PFOA (24 h or 24 + 8 d) were stained for senescence-associated β -galactosidase (SA- β -gal) activity using the Senescent Cells Staining Kit (Sigma, USA), according to the instructions provided by the manufacturer. The percentage of SA- β -gal-positive cells was calculated by counting 500 randomly selected blue interphase cells under bright field illumination.

2.7. Microscopy

Microscopy observations were carried out using a Leica DMI 3000B microscope (Germany), equipped with an EL6000 compact light source and appropriate excitation filters. The images were acquired with a CCD camera Leica DFC310FX and processed using the software Leica Application Suite 3.5.0 and Adobe Photoshop 9.0 (Adobe Systems Inc., USA). All comparative images (treated vs. untreated samples) were obtained under identical microscope and camera settings.

2.8. Data analysis

Statistical analysis was performed using SPSS Statistics 21 software (SPSS Inc., Chicago, IL, USA). The results were analyzed by Student's t-test for comparing paired samples and analysis of variance (ANOVA) with Bonferroni as *post hoc* test for multiple samples. Each data point represents the arithmetic mean \pm standard deviation of at least three independent experiments and 2-4 technical replicates. EC50 (50% effective concentration) values were obtained with GraphPad Prism 4.0 (GraphPad software, USA) using non-linear regression. Unless otherwise stated, differences were considered statistically significant at $p < 0.05$. Partial correlation coefficients (r) were

calculated to assess the association between the various measured parameters and considered significant at $p < 0.001$.

3. Results

3.1. PFOA decreases cell viability and impairs mitotic progression of HaCaT cells.

Concentration-response data and EC50 values for the different PFOA treatments in HaCaT cells are depicted in Fig. 1. The results of TPC assay that reflects the total number of cells showed statistically significant differences between treated and untreated control cultures at concentrations $\geq 100 \mu\text{M}$ in all three exposure conditions (24, 24+48 and 72 h). The MTT assay revealed that PFOA concentrations $\geq 250 \mu\text{M}$ decreased significantly cell viability following a 24 h exposure, a cytotoxic effect which continued 48 h after drug removal. The results of NRU assay indicated that HaCaT cells treated for 24 h with PFOA concentrations $\geq 100 \mu\text{M}$ reduced their viability in a concentration-dependent manner and showed a minor recovery after 48 h in drug-free medium. It should be noted that continuous PFOA incubations of 72 h consistently produced the highest cytotoxicity, with EC50 values around $100 \mu\text{M}$. In addition, the comparison of results obtained in all three assays with the different sequences of drug exposure showed a significant positive correlation ($p < 0.001$), according to Pearson analysis (Supplementary Table 1).

In parallel, mitotic indices were estimated to evaluate the possible interference of PFOA with the proliferative activity of HaCaT cells. The data summarized in Fig. 2 reveal that cell proliferation in treated monolayer cultures was altered, as indicated by the significant changes in the percentage of mitotic cells. Our results revealed that exposure to PFOA concentrations $\geq 100 \mu\text{M}$ cause a significant reduction in mitotic activity values, irrespective of the sequence of treatments (24, 24+48 and 72 h). On the other hand, continuous treatments with $50 \mu\text{M}$ PFOA (24 and 72 h) caused a significant accumulation of cells in mitotic stages prior to anaphase which was sustained after

drug withdrawal (24+48 h) and results in increased mitotic indices when compared to the respective controls. It is worth mentioning that immunofluorescence analysis of PFOA-treated cells revealed no evident alterations in spindle organization and/or chromosome congression under any of the tested experimental conditions (Supplementary Fig. S1). Considering the above results, we selected 50 μ M PFOA as the suitable concentration for all the subsequent long-term experiments.

3.2. PFOA causes cell cycle arrest after a post-exposure period of 8 days.

In the second set of experiments, cells pretreated for 24 h with 50 μ M PFOA were processed for flow cytometry analysis of DNA content, after a post-exposure period of 8 days (24+8 d). Our results revealed an evident deregulation of cell cycle progression in HaCaT cells, when comparing the profiles with the respective untreated control cultures (Fig. 3). A significant G0/G1 cell cycle arrest was observed, with a concomitant decrease in the proportion of cells in S and G2/M phases. In addition, a slight and non-significant increase in the fraction of polyploid cells was detected.

3.3. PFOA induces genotoxic damage that persists after a recovery period of 8 days.

To determine the possible mechanisms responsible for the alterations in cell cycle profiles, we first assessed the appearance of nuclear γ -H2AX foci, in the same experimental conditions described above. As shown in Fig. 4, the percentage of γ -H2AX-positive cells was significantly increased by 4.5-fold following a pretreatment with 50 μ M PFOA for 24 h, when compared with untreated control values. Moreover, it should be noted that a significant number of γ -H2AX positive cells, which was less (approximately half) than that observed immediately after PFOA exposure, was still present at the end of the recovery period.

HaCaT cells were also positive for 8-OHdG staining, a critical biomarker of oxidative stress. As shown in Fig. 5, cell cultures treated for 24 h with 50 μ M PFOA showed a

moderate enhancement of 8-OHdG levels which were only significantly increased 8 days after cessation of the treatment.

3.4. PFOA treatment withdrawal leads to induction of senescence markers in HaCaT cells.

Cells pretreated with 50 μ M PFOA and allowed to recover for 8 days (24+8 d) displayed morphological changes such as enlargement, flattening and increased cytoplasmic granularity, compatible with a senescent phenotype (Supplementary Fig. S2). To confirm these initial observations, HaCaT cells were processed in the same experimental conditions for SA- β -gal staining assay, a well-established indicator of senescence. PFOA treatment withdrawal resulted in an approximately threefold increase in the number of SA- β -gal-positive cells, compared to the respective controls (Fig. 6). In addition, positive immunofluorescence staining of IL-6, a cytokine involved in the development of senescence-associated secretory phenotype (SASP), was observed in a significant number of HaCaT cells (Fig. 7).

4. Discussion

It has been shown that the time elapsed between the end of an incubation period and the initiation of cytotoxicity assays can be a key factor to prevent underestimation of chemical-induced toxicity *in vitro* (McKim, 2010). However, no method has been established to accurately predict the potential long-term toxicity of chemicals at subcytotoxic concentrations, in terms of days or weeks after drug exposure (Toussaint et al., 2000). The current work was essentially designed to address this question since, to our knowledge, no previous *in vitro* studies have examined cytotoxicity recovery and/or possible induction of lasting toxic effects in PFOA-treated mammalian cells.

In our initial set of experiments, we sought to investigate PFOA-mediated cytotoxic effects in HaCaT cells and to determine their potential reversibility after a 48 h recovery period. Cell viability was reduced in a concentration- and time-dependent manner

following a 24 or 72 h continuous exposure to the compound, according to MTT and NRU assays. Furthermore, data from recovery experiments revealed that cytotoxic effects in response to PFOA treatments persist for at least 48 h after removing the drug. Remarkably, equivalent findings have been obtained in HeLa cells under the same experimental conditions (our unpublished results). It is worth mentioning that quantitative determinations of total protein content in HaCaT cultures, as an indirect method of estimating cell number, showed comparable data to those obtained with viability assays in both the continuous and recovery experiments. Therefore, the above results clearly suggest that PFOA mainly affects cell growth rather than cell viability, over the entire concentration range tested.

This assumption was further confirmed by the finding that growth inhibitory effects induced by high PFOA concentrations ($\geq 100 \mu\text{M}$) were associated with a significant and non-reversible decline in mitotic activity of HaCaT cells. By contrast, an increased fraction of cells in early mitosis was observed after the different treatments (24, 24+48 and 72 h) with a moderate concentration of the compound ($50 \mu\text{M}$). Noteworthy, cell division was successfully completed and spindle assembly disruption was not apparent in immunofluorescence analysis. Likewise, no significant rise in cell number was found in PFOA-treated cultures, as compared to the respective controls, thus indicating defective mitotic progression rather than stimulation of cell proliferation. Interestingly, despite some methodological differences, our results seem to be in good agreement with those reported recently using other human cell lines which consistently revealed that high PFOA concentrations cause cell cycle arrest in G0/G1 phase, while non-toxic concentrations increase the proportion of G2/M cells (Huang et al., 2013; Rainieri et al., 2017; Zhang et al., 2016).

Furthermore, the current work provides unprecedented evidence that a single exposure to this perfluorinated compound is sufficient to induce lasting proliferative changes in human normal cells. This finding suggests that impairment of HaCaT cells proliferation can develop within the first 24 h of drug exposure and was not restored

during the recovery period evaluated. However, since perfluorinated compounds, including PFOA, are known to readily accumulate and be retained in cultured mammalian cells (Sanchez Garcia et al., 2018), we cannot rule out that prolonged action of the drug during withdrawal could also explain our results. It should be noted that these assumptions are not mutually exclusive and future investigation is required to elucidate the relative contribution of each process.

In the second part of the study, the occurrence of long-term and/or delayed cytotoxic effects was compared with the short-term response of HaCaT cells subjected to a moderate treatment with PFOA (50 μ M, 24 h). It is well recognized that environmental and industrial chemicals may activate transient cell stress responses that largely depend on the nature and severity of the stimulus, but also on the cell type (Fulda et al., 2010). However, an immediate cell response does not exclude the induction of other delayed stress-triggered pathways that can lead to long-lasting or even permanent cellular damages (Smirnova et al., 2015).

Our results showed that HaCaT cells treated with 50 μ M PFOA for 24 h which exhibited a delay in mitotic stages prior to anaphase, as described above, also displayed a significant number of nuclear γ -H2AX foci, a sensitive marker of DNA double-strand breaks (DSBs) formation and repair (Sharma et al., 2012). DSBs can arise during DNA replication or endogenous cell metabolism but also from direct exposure to external factors, such as ionizing radiation and chemical agents (Chatterjee and Walker, 2017). These harmful DNA lesions must be efficiently repaired, through activation of a complex signaling network collectively termed DNA damage response (DDR), to prevent genome instability and carcinogenesis (Wilson and Durocher, 2017). However, it should be noted that p53-deficient cells like HaCaT keratinocytes, with mutations in both alleles of the p53 gene (Lehman et al., 1993), are predisposed to enter mitosis despite unrepaired or misrepaired DNA damage (Bakhom et al., 2017). This might explain, at least in part, the defective mitotic progression of PFOA-treated cells since persistent pre-mitotic damage or partial

induction of DDR in mitosis is known to perturb several aspects of cell division, including significant delays in the anaphase onset (Alcaraz-Silva et al., 2014; Mikhailov et al., 2002). Although most *in vivo* and *in vitro* assays have revealed no direct mutagenic or genotoxic risks associated with PFOA exposure (Butenhoff et al., 2014; US EPA, 2016), some limited findings on human tumor cells clearly indicate that the compound may induce DSBs as a result of increased reactive oxygen species (ROS) generation (Yahia et al., 2014; Yao and Zhong, 2005). Our results were not entirely consistent with this assumption because DNA damage found in HaCaT cells immediately after a moderate treatment with PFOA was not concomitant with significant oxidative stress, as determined by 8-OHdG immunostaining.

A further interesting observation in this study was that γ -H2AX foci were still present, although at reduced levels, in cells grown for up to 8 days (24+8 d) in PFOA-free complete medium. The persistence of residual γ -H2AX foci provides evidence for sustained activation of DDR and has been consistently interpreted as incomplete or defective repair of DNA damage (Olive, 2011; Siddiqui et al., 2015). Moreover, a considerable number of HaCaT cells exhibited cell cycle arrest at the G0/G1 phase, as revealed by flow cytometry, and displayed significant positive staining for acidic β -galactosidase, cytokine IL-6 and the oxidative stress marker 8-OHdG. Taken as a whole, these features are compatible with the so-called senescence-associated secretory phenotype (SASP) or senescence-messaging secretome (Coppé et al., 2008; Kuilman and Peeper, 2009) which includes the secretion of pro-inflammatory cytokines, chemokines, growth factors and proteases that depend on the cell type (Coppé et al., 2008; Davalos et al., 2010). Noteworthy, SASP markers in HaCaT cells were evident 8 days after cessation of PFOA exposure and undetectable at previous time points (data not shown). This observation is clearly consistent with findings of other authors revealing that SASP in cultured cells requires more than 5 days to develop, after DDR induction (Coppé et al., 2010). Therefore, it can be reasonably assumed that a moderate exposure to PFOA stimulates the appearance of SASP in HaCaT cells

through prolonged activation of DDR. In support of this assumption, secretion of IL-6, a key component of the pro-inflammatory SASP seems to be directly correlated with persistent but not transient DNA damage signaling (Rodier et al., 2009). Moreover, it is well documented that senescent cells generate increased ROS levels both *in vitro* and *in vivo*, although the precise underlying mechanisms are still controversial (Davalli et al., 2016; Nair et al., 2015). Several evidences based on different analytical techniques have demonstrated a direct binding of PFOA to DNA by hydrophobic interactions, resulting in destabilization of the secondary structure and subsequent damage (Lu et al., 2016; Zhang et al., 2009). Nevertheless, the ability of PFOA to cause localized and unreparable DNA lesions or repair machinery defects in human cells is a challenging question that requires further investigation.

Cellular senescence is a stress response characterized by a permanent cell cycle arrest that has emerged as a tumor suppressive mechanism but, depending upon the cellular context, senescence and the SASP can contribute to various biological processes with both beneficial or detrimental effects (Muñoz-Espín and Serrano, 2014; Rodier and Campisi, 2011). Despite that senescent cells were initially defined in the human skin (Dimri et al., 1995), the characterization and impact of the SASP in the diverse skin cell types, and particularly in keratinocytes and melanocytes, is still under debate (Touffaire et al., 2017). However, recent studies have demonstrated that the SASP promotes accurate wound healing but is also a critical effector in skin cancer and aging (Demaria et al., 2015; Ghosh et al., 2016; Touffaire et al., 2017). It should be pointed out that PFOA concentrations used in this study were several orders of magnitude above the serum levels found currently in the general population, with mean concentrations of 1.94 ng/mL (CDC 2018), but they are comparable to those reported in specific occupational settings, with mean (1052 ng/mL) and maximum (32000 ng/mL) serum concentrations corresponding to 2.5 and 77 μ M (Fu et al., 2016). Although it is difficult to directly compare *in vitro* data with *in vivo* exposure findings, the use of HaCaT cells widely accepted as a reliable model to assess human skin carcinogenesis

(Fusenig and Boukamp, 1998) as well as inflammatory/repair responses of human keratinocytes (Colombo et al., 2017), emphasizes the relevance of our results.

In conclusion, this paper provides novel data and insights regarding the cytotoxic effects caused by PFOA in human cells and highlights the importance of considering both the immediate and delayed cellular responses, to ensure the accurate identification of chemical toxicity in cell-based systems. Further studies are underway to verify whether the PFOA-mediated induction of senescence phenotype, described in this study, is cell-type-specific or consistently found in other non-tumoral human cell lines.

Acknowledgments

This work was supported by the Spanish Ministry of Economy and Competitiveness [grant number CTM2012-31344]. The authors thank Dr. J.M. Pérez Martín for valuable collaboration.

Conflict of interest

The authors declare no competing or financial interests.

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Figure captions

Fig. 1. Cytotoxic effects in HaCaT cells after treatment with increasing PFOA concentrations. Total protein content (TPC), Neutral red uptake (NRU) and MTT reduction values immediately after a 24 h exposure, after a 24 h exposure + 48 h recovery period in drug-free medium, and after a 72 h continuous exposure. The data are expressed as a percentage of respective controls and represent the mean \pm standard deviation of three independent determinations. Lower table shows EC50 values for all the experimental conditions. Asterisks indicate statistically significant differences between treated cells and untreated control cultures (ANOVA and Bonferroni post hoc test, $p < 0.05$).

Fig. 2. Mitotic alterations in HaCaT cells after treatment with increasing PFOA concentrations. Mitotic index values following a 24 h exposure (upper panel), 24 h + 48 h recovery period in drug-free medium (middle panel), and after a 72 h continuous exposure to the compound (lower panel). The data are expressed as a percentage of control and represent the mean \pm standard deviation of three independent determinations. Black areas of the charts show the fraction of cells in mitotic stages prior to anaphase and gray areas those in anaphase or telophase. Asterisks indicate statistically significant differences between treated cells and untreated control cultures (ANOVA and Bonferroni post hoc test, $p < 0.05$).

Fig. 3. Cell cycle profiles of HaCaT cells following a treatment with 50 μ M PFOA for 24 h and a post-exposure period of 8 days. Flow cytometry data of DNA content are expressed as a percentage of cells analyzed and represent the mean \pm standard deviation of at least three independent experiments. Asterisks indicate statistically significant differences between treated cells and untreated control cultures (Student's t test, $p < 0.05$).

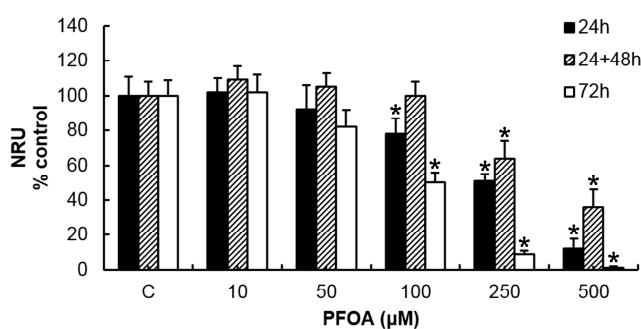
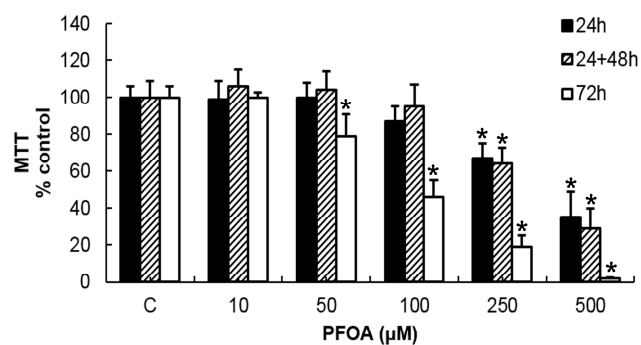
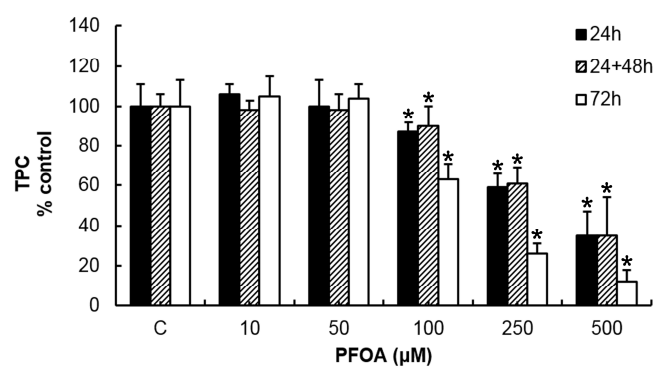
Fig. 4. Generation and persistence of double strand breaks in PFOA-treated HaCaT cells. γ H2AX-positive cells immediately after a 24 h exposure to 50 μ M PFOA and following a post-exposure period of 8 days in drug-free medium. The data are expressed as percentage of that found in respective control cultures and represent the mean \pm standard deviation of three independent experiments. Statistically significant differences between treated and untreated control cells were assessed by the Student's t test (* $p < 0.05$; *** $p < 0.001$).

Fig. 5. Evaluation of ROS generation in PFOA-treated HaCaT cells. Cells with positive 8-OHdG staining immediately after a 24 h exposure to 50 μ M PFOA and following a post-exposure period of 8 days in drug-free medium. The data are expressed as percentage of that found in respective control cultures and represent the mean \pm standard deviation of three independent experiments. Asterisks indicate statistically significant differences between treated cells and untreated control cultures (Student's t test, $p < 0.05$).

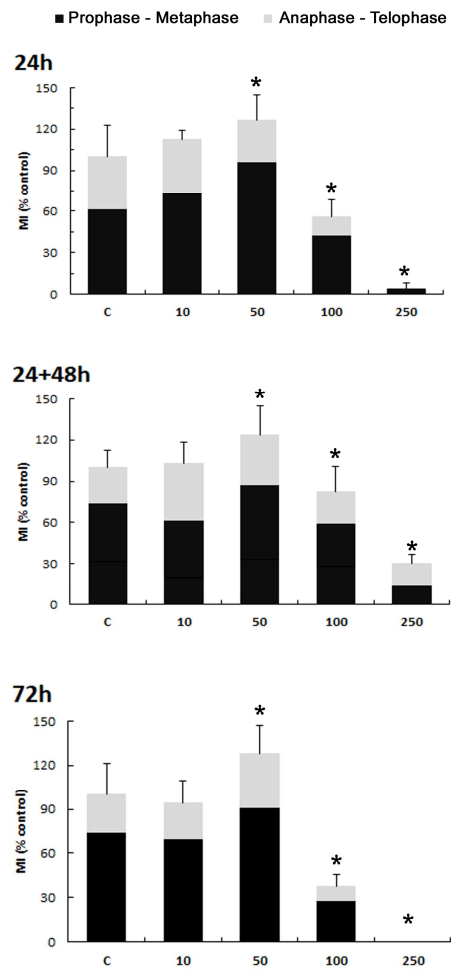
Fig. 6. Identification of senescence in PFOA-treated HaCaT cells. Cells with positive staining for senescence-associated- β -galactosidase activity immediately after a 24 h exposure to 50 μ M PFOA and following a post-exposure period of 8 days in drug-free medium. The data are expressed as percentage of that found in respective control cultures and represent the mean \pm standard deviation of three independent experiments. Asterisks indicate statistically significant differences between treated cells and untreated control cultures (Student's t test, *** $p < 0.001$). Right panel shows representative images of β -galactosidase positive (upper) and negative (lower) cells. Bar : 10 μ m.

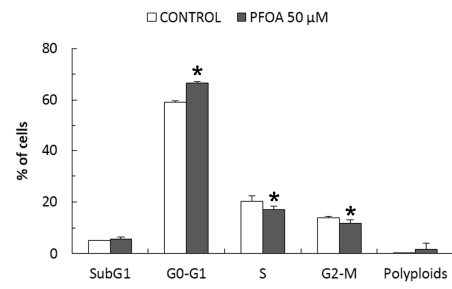
Fig.7. Detection of IL-6 secretion in PFOA-treated HaCaT cells. (A) Quantification of cells with positive immunofluorescence staining for IL-6 in untreated control cells and

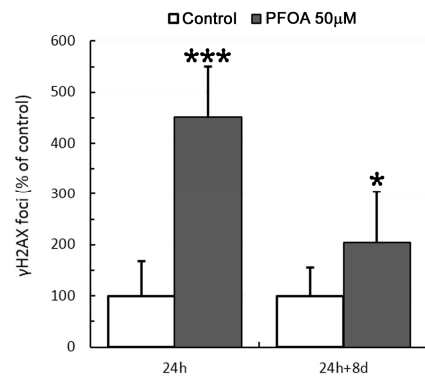
after treatment of cells with 50 μ M PFOA for 24 h followed by a post-exposure period of 8 days in drug-free medium. The data represent the mean \pm standard deviation of three independent experiments and asterisks indicate statistically significant differences between treated and untreated control cells (Student's t test, *** $p < 0.001$). (B) Representative immunofluorescence images of IL-6 positive cells counterstained with Hoechst 33258. Bar: 10 μ m.

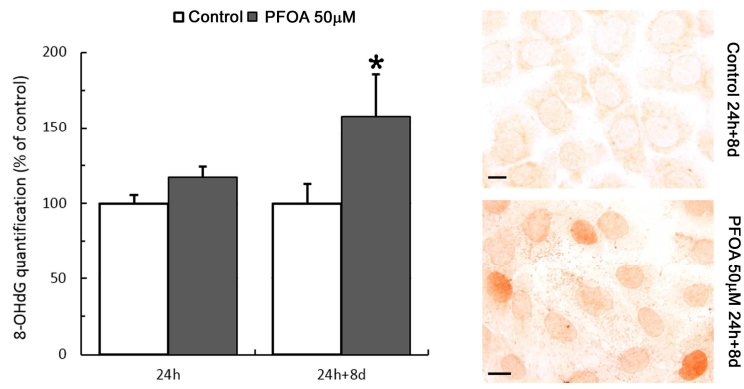


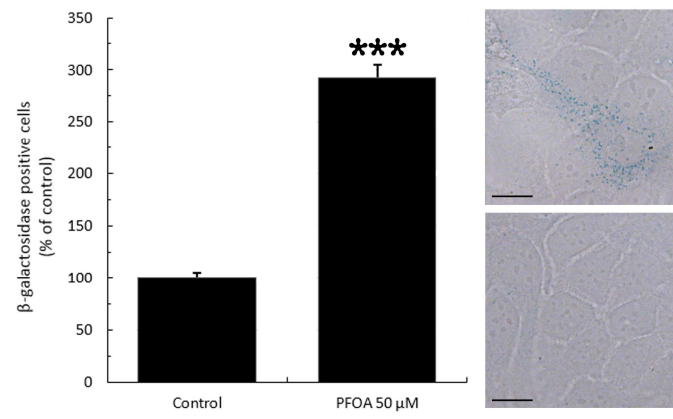
| EC50 (μM) | 24h | 24+48h | 72h |
|-----------|--------------|---------------|--------------|
| TPC | 210.3 ± 35.1 | 237.85 ± 26.4 | 100.51 ± 1.0 |
| MTT | 261.1 ± 86.1 | 269.0 ± 96.9 | 103.5 ± 39.6 |
| NRU | 231.3 ± 27.1 | 241.2 ± 89.2 | 109.1 ± 9.9 |

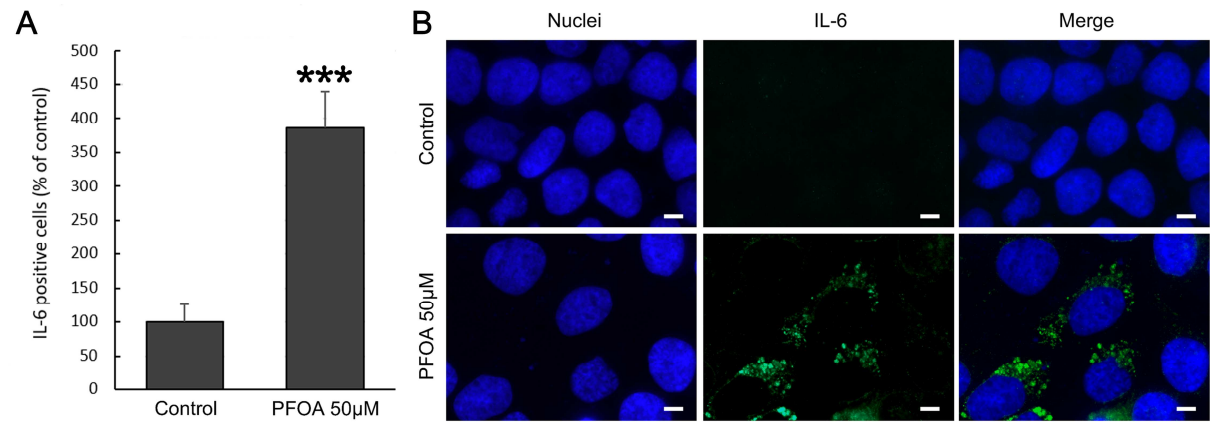












Highlights

- PFOA exposure mainly affects cell growth rather than viability of HaCaT cells.
- A persistent DNA damage response determines the long-term fate of PFOA-treated cells.
- DSBs caused by a mild treatment with PFOA may not be attributed to oxidative stress.
- HaCaT cells pretreated with PFOA develop senescence-associated secretory phenotype.