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Bisphenols and perfluoroalkyls alter human stem cells integrity: A possible link with infertility

Giulia Gaggi [a,b,c,1], Andrea Di Credico [a,b,c,1], Federica Barbagallo [d], Barbara Ghinassi [a,b,c,*], Angela Di Baldassarre [a,b,c]

[a] Reprogramming and Cell Differentiation Lab, Center for Advanced Studies and Technology (CAST), 66100, Chieti, Italy
[b] Department of Medicine and Aging Sciences, "G. D'Annunzio" , University of Chieti-Pescara, 66100, Chieti, Italy
[c] UdA -TechLab, "G. D'Annunzio", University of Chieti-Pescara, 66100, Chieti, Italy
[d] Faculty of Medicine and Surgery, Kore University of Enna, Enna, Italy

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ABSTRACT

Bisphenols and Perfluoroalkyls are chemical compounds widely used in industry known to be endocrine disruptors (EDs). Once ingested through contaminated aliments, they mimic the activity of endogenous hormones leading to a broad spectrum of diseases. Due to the extensive use of plastic in human life, particular attention should be paid to antenatal exposure to Bisphenols and Perfluoroalkyls since they cross the placental barrier and accumulates in developing embryo. Here we investigated the effects of Bisphenol-A (BPA), Bisphenol-S (BPS), perfluorooctane-sulfonate (PFOS) and perfluorooctanoic-acid (PFOA), alone or combined, on human-induced pluripotent stem cells (hiPSCs) that share several biological features with the stem cells of blastocysts. Our data show that these EDs affect hiPSC inducing a great mitotoxicity and dramatic changes in genes involved in the maintenance of pluripotency, germline specification, and epigenetic regulation. We also evidenced that these chemicals, when combined, may have additive, synergistic but also negative effects. All these data suggest that antenatal exposure to these EDs may affect the integrity of stem cells in the developing embryos, interfering with critical stages of early human development that might be determinant for fertility. The observation that the effects of exposure to a combination of these chemicals are not easily foreseeable further highlights the need for wider awareness of the complexity of the EDs effects on human health and of the social and economic burden attributable to these compounds.

1. Introduction

According to the "One Health" vision, recognized by the European Commission and in the Green Deal, the health of humans, animals and the environment are inextricably linked, and must be preserved with a "Green" approach. Consequently, the study of contaminants leached from plastic that pass from the environment to food, altering ecosystems and the health of living organisms, is very relevant. Bisphenols (BPs), such as BPA and BPS, and Perfluoroalkyles (PFs), such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), are plastic pollutants leached by many everyday products that belong to the endocrine disruptors (EDs), a family of compounds that might interfere with hormones inducing health consequences (Goldstone et al., 2015)–(Shafei et al., 2018). Evidence on the EDs effects on human health mainly comes from studies on single molecules, but humans are simultaneously daily exposed to cocktails of a wide variety of different chemicals, which may have additive/synergistic biologic effects: this results in a big gap between current studies and real exposure effects (Maqbool et al., 2016).

Pregnancy is one of the most susceptible periods to plastic pollutants: BPs and PFs can cross the placental barrier and compromise early developmental processes. BPA can cause alterations in the reproductive organs of developing mice embryos (Schönfelder et al., 2002) BPS, often used as an alternative to BPA because leaches to a lesser degree from plastics was detected at greater concentrations than BPA in human samples and in both maternal and fetal plasma (Zhao et al., 2018), (Pan et al., 2020). PFOS, widely used in industrial products, accumulates in the liver where alters lipid metabolism and hormone regulation; being

* Corresponding author. University of Chieti-Pescara, Department of Medicine and Aging Sciences, Via dei Vestini 31, 66100 Chieti, Italy.
E-mail address: b.ghinassi@unich.it (B. Ghinassi).
[1] Equal contribution.

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very stable in the environment, it has been also detected in breast milk, maternal serum, and cord blood (Chen et al., 2012), (LaKind et al., 2022). Prenatal exposure to PFOA, another very stable contaminant, is associated with lower birth weight (Peterson et al., 2022). EDs have been also detected in human follicular fluid, which surrounds and nourishes developing eggs within the ovaries (Liang and Zhang, 2013): while the exact implications of this preconceptional contamination are far to be fully understood, given the potential for EDs to disrupt hormonal signaling, the possibility that the presence of xenobiotics in the follicular fluid might affect the reproduction cannot be ruled out. Despite the growing evidence of the detrimental health effects deriving from preclinical and association studies, only limited data are available about embryo susceptibility to BPs and PFS due to ethical issues related to the use of human embryonic models. These problems have been partially overcome when Yamanaka et al. generated human pluripotent stem cells (hiPSCs) from adult cells through the forced expression of *OCT3/4, SOX2, KLF4*, and *c-MYC*: due to the activity of these factors, differentiated cells can be reprogrammed and returned to a pluripotent state, comparable to human embryonic stem cells (hESCs) in terms of transcription programs and global chromatin configuration (Liang and Zhang, 2013)– (Gaggi et al., 2020). Thanks to this genetical reprogramming, indeed, hiPSCs acquire an ESC -like state, characterized by the ability to undergo self-renewal indefinitely and to differentiate into cells from all three germ layers (Di Baldassarre et al., 2018a), (Di Credico et al., 2021): in this way, they represent a valuable surrogate of blastocyst cells being morphologically, molecularly and epigenetically almost indistinguishable from ESCs (Parrotta et al., 2017).

Aim of this study is to provide evidence on the developmental toxicity of the plastic pollutants BPs and PFs, as a single molecule or in combination, by analysing their effects on human stem cell integrity. Due to the ethical restriction in the use of human embryonic stem cells, we exploited hiPSCs that, being comparable to stem cells that constitute the inner cells mass of the blastocyst, may be considered as an *in vitro* model of cells of developing embryos.

2. Material and methods

2.1. Human cell cultures and treatments

Normal hiPSCs were purchased from iXCells Biotechnologies (San Diego, USA) and cultured on Matrigel (Corning, Sommerville, MA, USA) pre-coated 6 well plates (Corning Sommerville, MA, USA) in mTeSR Plus medium (STEMCELL Technologies, Vancuver, Canada), following the manufacturer's procedures.

BPA, BPS, PFOS or PFOA (Wellington Laboratories, Guelph, Canada) dissolved in methanol were serially diluted in culture media. Cells were then exposed to 0.1 μM of BPA, BPS, PFOS or PFOA individually, or to a cocktail in which EDs were paired or all in combination (BPA + PFOS, BPA + PFOA, BPS + PFOS, BPS + PFOA, BPA + BPS, PFOS + PFOA, BPA + BPS + PFOS + PFOA).

2.2. Analysis of impedance

HiPSCs were seeded at 15–20 $\times$ 10^5 on 96-Well CytoView MEA plate (Axion Biosystem, Atlanta, GA, USA). When the cells were in the logarithmic growth phase, samples were treated with BPA, BPS, PFOS or PFOA 0.1 μM, alone and in combinations. The effects of the EDs on cell growth rate and viability were monitored in real-time measuring the cell impedance (ohms, Ω) by means of microelectrodes embedded in the culture surface of each well: cells growth was detected as an increase in impedance, while cell death or detachment as impedance decrease; hiPSCs were treated with the pollutants, individually or in combination, and continuous impedance data have been registered up to 48 h to evaluate the cell-drug responses over time by Maestro Edge (Axion Biosystem, Atlanta, USA) using the Impedance Module of Axion Integrated Studio software (Axion Biosystem, Atlanta, USA). The resistance was set at 41.5 kHz.

2.3. Immunofluorescence analysis of Ki67 expression

HiPSCs were seeded at 15–20 $\times$ 10^5 cell/well in μ-Slide 8 Well (Ibidi, Germany) precoated with Matrigel 1:50. During the exponential phase, cells were treated with 0.1 μM of BPA, BPS, PFOS, and PFOA, as single molecule or combined, for 24 h. Then immunofluorescence analysis was performed as previously described (Gaggi et al., 2022a). Briefly, cells were fixed in paraformaldehyde 4% and permeabilized by Triton 0.5%. After blocking with BSA 5%, cells were stained with anti-Ki67 antibody 1:100 Alexa fluor 488 conjugated (Thermo Fisher Scientific) overnight at 4 °C. Nuclei were counterstained with DAPI 1:1000 (Thermo Fisher Scientific). Pictures were acquired by EVOS M7000 (Thermo Fisher Scientific) and analyzed by Celleste Image Analysis Software (Thermo Fisher Scientific).

2.4. Immunofluorescence analysis of mitochondrial membrane potential (MMP)

The mitochondrial functionality was analyzed by HCS Mitochondrial Health Kit (Thermo Fisher Scientific) following the manufacturer's procedures. HCS Mitochondrial Health Kit contains a specific fluorescent probe that stores inside active and healthy mitochondria, that then appear brilliantly red; changes in the mitochondrial membrane potential (MMP) are detected as modification of the staining intensity. Briefly, hiPSCs were treated with 0.1 μM of BPA, BPS, PFOS, and PFOA individually or in combinations for 24 h; cells were then incubated with the MitoStain for 30 min at 37 °C and then fixed in paraformaldehyde 4% for 10 min; nuclei were counterstained by Hoechst 33,342. To quantify the fluorescence, 9 different fields for each sample were acquired by EVOS M7000 (Thermo Fisher Scientific) and analyzed by Celleste Image Analysis Software (Thermo Fisher Scientific).

2.5. RNA extraction and reverse transcription

HiPSCs, treated with different combination of EDs for 24 h, were lysed with QIAzol lysis reagent (QIAGEN, Hilden, Germany) and total RNA was extracted using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's procedure. For reverse transcription, 1 μg of RNA was retrotranscribed by the High-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) following to the manufacturer's procedure.

2.6. Real time PCR (qPCR)

For all the examined mRNAs, qPCR analysis was performed using SYBR green (PowerUp SYBR Green Master mix, Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Gaggi et al., 2022b) in QuantStudio 3 (Thermo Fisher Scientific Waltham, MA, USA). The run method consisted of the following steps: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min. Steps 2 and 3 were repeated for 40 cycles. The authenticity of the PCR products was verified by melt-curve analysis. Each gene expression value was normalized to the *18S* expression. The fold change of each gene was calculated by the ΔΔCT method and expressed in relation to the CTRL. Primer sequences are listed in Table 1.

2.7. Statistical analysis

All data are presented as the mean ± SD of 3 independent experiments. The statistical analysis was performed by Prism 9 (Graphpad, San Diego, CA, USA) using the Student's T-Test. The level of significance was set at $p < 0.05$.

3. Experimental design

The potential BPs and PFs developmental toxicity have been evaluated by treating the hiPSCs with 0.1 μM of BPA, BPS, PFOS and PFOA. Cells were exposed to a single chemical, or to a cocktail in which EDs were paired or all in combination: BPA + PFOS, BPA + PFOA, BPS + PFOS, BPS + PFOA, BPA + BPS, PFOS + PFOA, and BPA + BPS + PFOS + PFOA (All). The effects of the exposure on cell proliferation, mitochondria health and on pluripotency, germinal specification, and epigenetic regulator genes were then evaluated (Fig. 1).

4. Results

4.1. hiPSCs proliferation rate is altered by exposure to a combination of BPs and PFs

We monitored the effects of the EDs on cell growth rate and viability measuring the cell impedance in real-time and we found that exposure to a single EDs did not affect the proliferation rate; when a combination of chemicals was applied, no modification of the proliferation was detected, except in BPA + BPS and when EDs were administrated all together: in these samples a proliferative surge-in was observed after 36–48 h of exposure, with an increment of the impedance values of about 20% compared to the control (Fig. 2 and Table 2). On the other side, the combination BPS + PFOS caused a dramatic reduction of the impedance, which become evident early after the treatment.

The EDs effects on the proliferative activity were also immunocytochemically evaluated by the expression of the nuclear protein Ki67, a well-recognized marker of proliferation highly expressed in G2-M phases of the cell cycle (Sobecki et al., 2016). As reported in Fig. 3, no difference in the percentage of $Ki67^{+}$ nuclei were detected after 24hr exposure to EDs, alone or mixed, except in BPS + PFOS sample, where we assisted in a dramatic reduction of Ki67 expression that confirmed the toxic effect of this chemical combination.

These results indicate that even if BPs and PFs administered individually seem not to interfere with the hiPSCs proliferative regulation, the combinations BPA + BPS and the cocktail of all the EDs drive the cells toward proliferation, while BPS + PFOS appeared toxic.

4.2. BPs and PFs, alone and in combination, exert a mitotoxic effect in hiPSCs

We investigated if BPs and PFs could be detrimental for mitochondria activity and metabolism and we observed that the 24-h exposure to the pollutants, administered singularly or in combination, affected the mitochondrial membrane potential (MMP), as reported in Fig. 4. In line with the toxic effect registered by the impedance analysis, the most dramatic result was observed when the cells were exposed simultaneously to BPS and PFOS. Unexpectedly, no modifications of MMP were detected in samples exposed to a combination of EDs belonging to the same family (BPS + BPA or PFOS + PFOA).

4.3. BPs and PFs exposure deeply affects the biological characteristics of hiPSCs by modifying the expression of genes related to pluripotency, germ line specification, and to the regulation of the epigenetic machinery

We then analyzed whether the BPs and PFs exposure interferes with the biological profile of hiPSCs by studying the possible modifications on genes involved in the maintenance of the pluripotency, in the germ cell specification, and in the epigenetic regulation.

We first performed the evaluation of the hiPSCs stemness characteristics by the qPCR analysis of NANOG, OCT4, SOX2, ESG1, KLF4 and REX1, markers that with their expression define the pluripotency of the cells. As shown in Fig. 5 and in Table 3, we found that BPs and PFs treatment, singularly or in combination, affected the stemness of hiPSCs, even if to a different extent. In particular, NANOG expression resulted upregulated by PFOS and PFOA when administered alone or combined respectively with BPS and BPA, while the concomitant exposure to PFOS and PFOA was ineffective. BPA and BPS seemed not to interfere with NANOG expression. Similarly, OCT4 was upregulated by the two PFs administered singularly or when PFOS was combined with both BPA and BPS. No effects were detected after treatment with BPs alone, in combination with PFOA and when EDs from the same chemical class (BPA + BPS and PFOS + PFOA) were administrated together. A slight reduction of OCT4 was detected cells were exposed to a cocktail of the 4 EDs. SOX2 resulted not affected by the pollutant exposure, except in BPS + PFOA sample in which an increase of SOX2 gene expression was measured. ESG1 was dramatically affected by the different treatments: indeed, ESG1 expression was highly up regulated hiPSCs treated with BPs and PFs singularly or in combination. Again, no effects on ESG1 gene expression were detected in the samples exposed to two EDs of the same class (BPA + BPS and PFOS + PFOA) or to the cocktail of the 4 compounds. Similarly, KLF4 expression was highly upregulated in all the experimental conditions, except for BPA + BPS sample. Finally, we tested the expression of REX1 whose expression, unlike the other stemness markers, was downregulated by EDs; indeed, BPA, alone or in combination with PFs, importantly decreased REX1 transcript levels; on the other side, BPS modified the REX1 expression when administered alone, but not when was combined with the two PFs. The cocktail of the four different EDs was effective in downregulating REX1 gene transcription, while the pairing of EDs of the same class (BPA + BPS and PFOS + PFOA) was not (see Table 3).

As hiPSCs express germ lineage markers (Yao et al., 2022), we then checked the possible effects of the EDs on genes specifically involved in different steps of the germline specification (Fig. 5 and in Table 3). BLIMP1(also known as PRDM1) (Ohinata et al., 2005), AP2γ (also

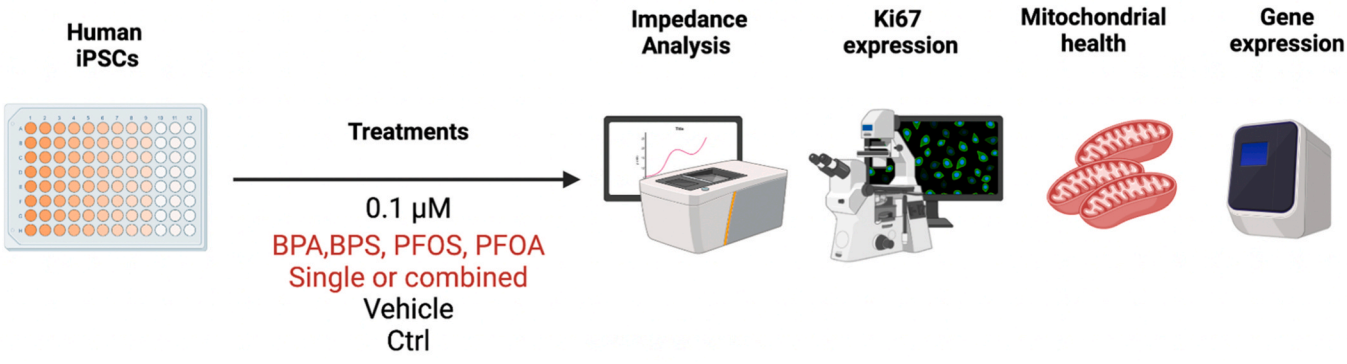


Fig. 1. Scheme of the experimental design.

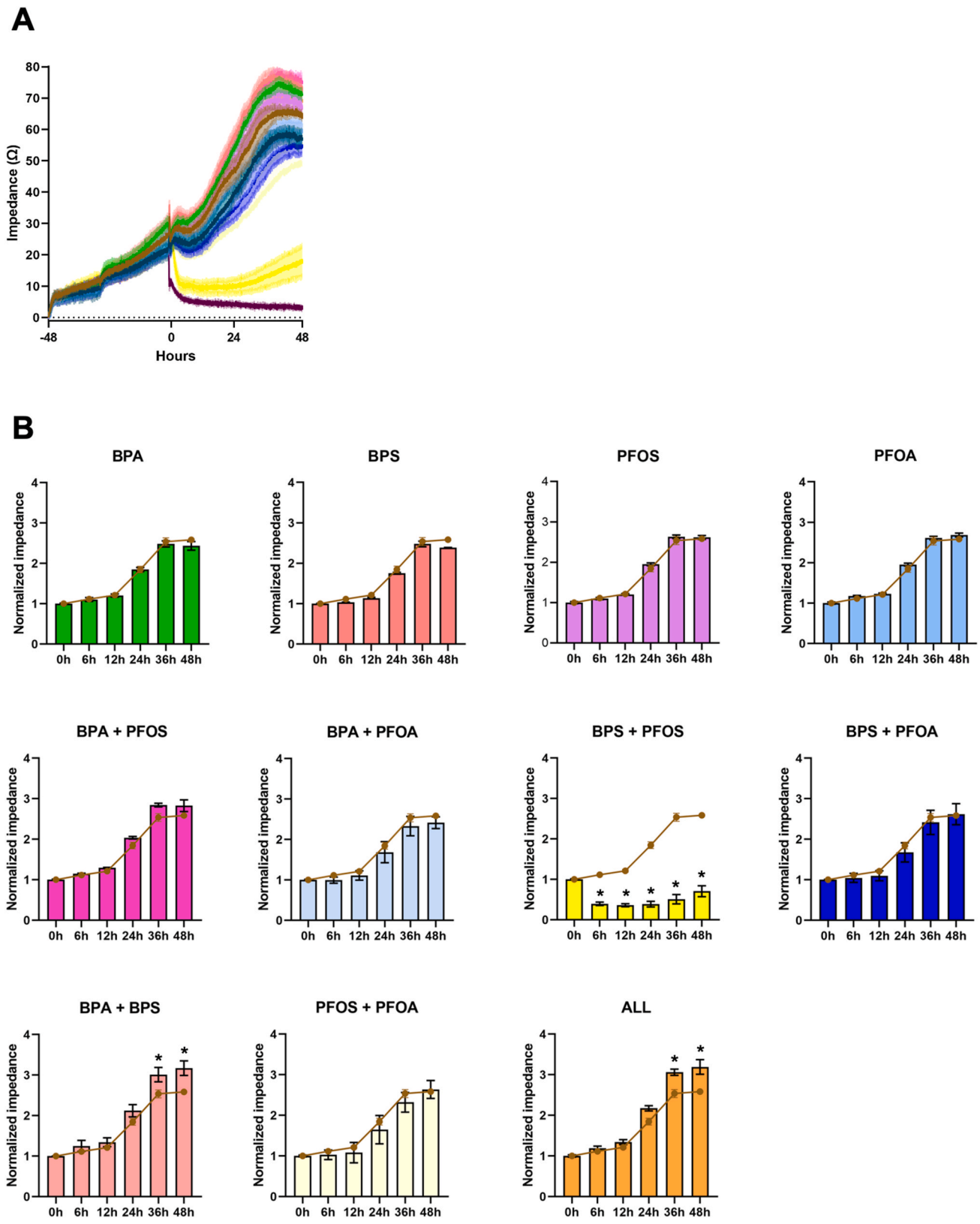


Fig. 2. Analysis of PBs and PFs effects on hiPSCs proliferation by impedance measurements. On day 0 hiPSCs were treated with 0.1 μM of BPA, BPS, PFOS or PFOA alone or in combination and the impedance values were monitored in real-time up to 48 h. Cells not treated (brown line), treated only with the vehicle (0.09% methanol) (dark blue line) or with lysing agents (purple line) represented the experimental controls. *Line graph panel*: absolute impedance values (expressed in ohms, Ω). Graph is representative of three different experiments. *Histogram Panels*: Normalized impedance values (Day 0 = 1) of Control (brown line) or samples treated with 0.1 μM of BPA, BPS, PFOS or PFOA alone or in combination, as indicated. Data are expressed as mean ± SD ($n = 3$, *$p < 0.05$ vs Control).

Table 1
This table includes the primer sequence for qPCR.

Gene	Sequence (5′ to 3′)	Reference
NANOG_FW	CCAGACCCAGAACATCCAGTC	10.3390/cells9051304
NANOG_REV	CACTGGCAGGAGAATTTGGC	
OCT4_FW	GGGTTTTTGGGATTAAGTTCTTC	10.1038/nbt.1503
OCT4_REV	GCCCCCACCCTTTGTGTT	
SOX2_FW	CAAAAATGGCCATGCAGGTT	10.1038/nbt.1503
SOX2_REV	AGTTGGGATCGAACAAAAGCTATT	
ESG1_FW	CCATGAATGCCCTCGAACTAGG	Gaggi et al., 2020
ESG1_REV	CCTTAACTCTTTAGGCTGGAGCA	
KLF4_FW	AGCCTAAATGATGGTGCTTGGT	10.1038/nbt.1503
KLF4_REV	TTGAAAACTTTGGCTTCCTTGTT	
REX1_FW	GCGCAATCGCTTGTCCTCAG	Gaggi et al., 2020
REX1_REV	CACATTCCGCACAGACGTGG	
STELLA_FW	CGTCGAGAGTCTGTAGGAGC	Primer Blast
STELLA_REV	GGCTCCTTGTTTGTTGGTCT	
FRAGILIS_FW	CCGTGAAGTCTAGGGACAGG	Primer blast
FRAGILI_REV	TGCCTCCTGATCTATCGCTGG	
OVOL1_FW	AGAGCAGAGACCATGGCTTC	10.3727/096368914X678553
OVOL1_REV	GACGTGTCTCTTGAGGTCGA	
BLIMP1_FW	GGAGAATGTGGACTGGGTAGAG	Primer Blast
BLIMP1_REV	GGAGTTACACTTGGGGGCAG	
PWIL2_FW	ACAGCAAGATGGCAGAGACC	Primer Blast
PWIL2_REV	GACGGTGTGCTGAAGGTACA	
AP2γ_FW	CGCTCATGTGACTCTCCTGAC	Primer Blast
AP2γ_REV	GGCCGCCAATAGCATGTTCT	
PRDM14_FW	TGAGCCTTCAGGTCACAGAG	Primer Blast
PRDM14_REV	TTTCCTATCGCCCTTGTCCACA	
DNMT1_FW	GCCAGAGATAGAGATCAAGCTG	Primer blast
DNMT1_REV	CACAGCGTGTCAGAGATGCC	
DNTM3A_FW	CCATCGTCAACCCTGCTCG	Primer blast
DNMT3A_REV	CACCACATTCTCAAAGAGCCAG	
DNTM3B_FW	GACTCGTTCAGAAAGCCCAG	Primer blast
DNMT3B_REV	GGACTCGTCCACATGGTTGC	
TET1_FW	ACTCCCTGAGGTCTGTCCTG	Primer blast
TET1_REV	CAGGTAGGGCTGCATGACTT	
TET2_FW	CTCAGCAGCAGCCAATAGGA	Primer blast
TET2_REV	CTGTCTGGCAAATGGGAGGT	
TET3_FW	AACTGCTCACTCAGCTCTGC	Primer blast
TET3_REV	GCAGCCCTCAGAAAAGGGAT	
18S_FW	CATGGCCGTTCTTAGTTGGT	10.3389/fgene.2020.627007
18S_REV	CGCTGAGCCAGTCAGTGTAG	

known as TFAP2C) (Pauls et al., 2005) and PRDM14 (Yamaji et al., 2008), the three transcription factors critical for primordial germ cell (PGC) specification (Magnúsdóttir et al., 2013), were differently affected by the treatments: in particular, BLIMP1 and AP2γ were modified in an opposite way (up- and down-regulated, respectively) only by the BPA exposure, while PRDM14 was downregulated from BPA, BPA + PFOA, BPS + PFOA and from all the EDs combined together. STELLA, a small protein predominantly expressed in germ cells, early embryos, and pluripotent cells that plays significant roles in epigenetic modifications (Zhao et al., 2019), (Li et al., 2018), responded to the treatments as REX1, as it was reduced by BPA and BPS used singularly, or when BPA was combined with PFs (PFOS or PFOA); the combination of BPS with the PFs did not induce any changes in STELLA expression, the mix of all the four EDs combined reduced this marker expression as well, but, again, no differences were detected when two EDs from the same class were combined together. FRAGILIS, another germline specifier differently involved in the developmental stages (Lange et al., 2003), was only barely affected by the EDs exposure, as its expression resulted altered only in PFOS and BPA + PFOA samples, in which a dramatic reduction of the transcript was observed. OVOL1, a marker linked to the activation of spermatocyte-specific genes (Siebert-Kuss et al., 2023), appeared diversely regulated by the pollutants, as it was highly upregulated by PFOS, whereas BPA, PFOA, BPA + PFOS and BPS + PFOA reduced its expression. We finally checked for PIWIL2, a germline specifier essential for spermatogenesis: this marker was downregulated from BPs, alone and in combination with PFs, with the only exception of BPS + PFOS; the same effect was observed when hiPSCs were exposed to the cocktail containing all the EDs. No changes in PIWIL2 expression were detected in PFOS, PFOA, BPA + BPS and PFOS + PFOA samples.

Finally, we analyzed the expression of molecules involved in epigenetic regulation, which is pivotal in the first stages after fertilization. The DNA methyltransferases (DNMT) are three enzymes that add a methyl group to CpG and non-CpG dinucleotide sites: DNMT1 is essential for the maintenance of methylation and chromatin stability, and DNMT3A and DNMT3B act as de novo methyltransferases, and are important for DNA methylation in the early embryonic stages (Uysal et al., 2017). As reported in Fig. 5 and in Table 3, while *DNMT1* was only scarcely affected by the treatments, being modified only by BPS, *DNMT3A* and *DNMT3B* were upregulated by BPS alone and downregulated by BPA + PFOA. In addition, we detected an increment of *DNMT3A* after the treatment with PFOA and BPS + PFOS, while *DNMT3B* increased in BPA + BPS sample, and decreased when hiPSCs were treated with all the EDs together. Ten-eleven translocation (TET) methylcytosine dioxygenases mainly demethylate DNA at regulatory regions and are essential in regulating cell fate during development and in embryonic stem cells by maintaining pluripotency or by regulating differentiation; they include 3 members: TET1 and TET2, whose expression increases during pre-implantation development, and TET3, which is highly expressed in oocytes and fertilized zygotes (Yang et al., 2020). We found that *TET1* was downregulated only when the hiPSC were treated with BPS and BPS + PFOA, whereas *TET2* was upregulated by BPA, BPS and BPA + PFOS; on the other hand, we found that almost all treatments modified *TET*3 expression, although with a different trend (up- or down-regulation): *TET*3 levels remained comparable to the control only when two EDs of the same class were combined (BPA + BPS and PFOS + PFOA) and in BPS + PFOA sample (Fig. 5 and in Table 3).

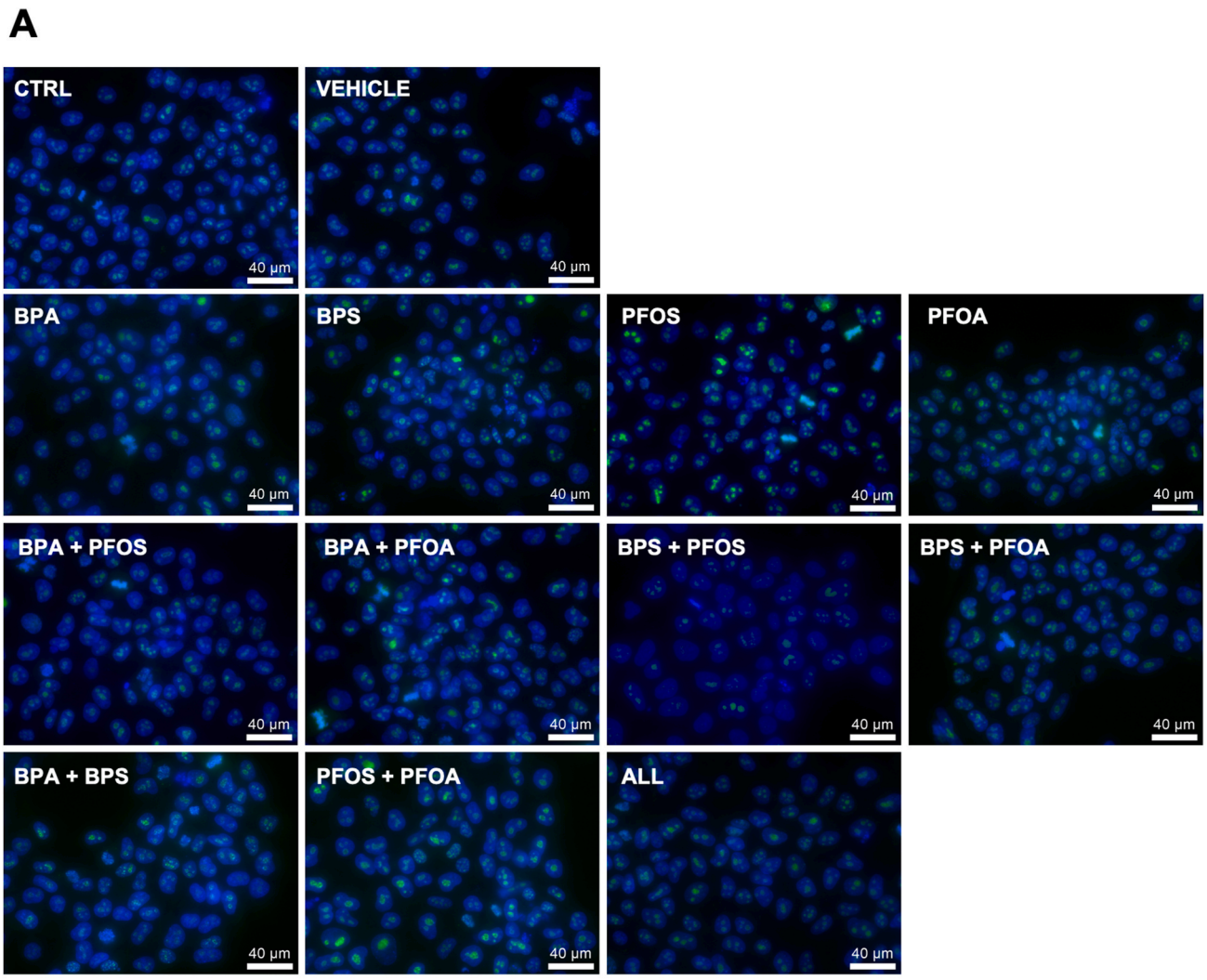
All results are summarized in Table 3.

5. Discussion

The main findings of this study are that *i.* the combined exposure to BPS and PFOS is highly toxic for hiPSC; in all the other conditions tested, BPs and PFs do not or only slightly alter the proliferative rate of hiPSCs, but deeply impact their metabolic status modifying the MMP; moreover, they may severely modify the biological characteristic of hiPSC impacting the expression of stemness markers, germline identifiers, and epigenetic regulators; overall, these data strongly suggest the potential developmental toxicity of BPs and PFs; *ii.* the effects elicited by a cocktail of the different EDs are not foreseeable as BPs and PFs do not have always additive or synergistic effects, but may also counteract each other; moreover, the combined exposure to substances of the same class (BPA + BPS or PFOS + PFOA) seems to be less detrimental than the exposure to the single chemical.

Health concern is rising about the immediate and long-term consequences of human exposure to plastic pollutants, that contaminate the environment releasing chemicals such as BPs and PFs, known to be EDs. As all humans, also pregnant women are unavoidably and unintentionally exposed to BPs or PFs using daily products. Pregnancy is a clinically relevant susceptible period for studying EDs exposures, because the xenobiotics may modulate physiologic processes not only in the exposed individuals but also in their offspring (Rolfo et al., 2020)–(Fouyet et al., 2022). Ethical and methodological issues hamper the study of the effects of human antenatal exposure to EDs; for this reason, we employed as cellular model the hiPSCs which are comparable, even if with some limitations, to the stem cells that constitute the inner cell mass of the blastocyst (Liang and Zhang, 2013), (Gaggi et al., 2021), (Gaggi et al., 2019).

Experimental studies frequently employ high doses of BPs or PFs, which may mirror an occupational exposure to EDs that is significantly higher than that of the general population (Lucas et al., 2023), (Ribeiro et al., 2017); moreover, combinations of EDs often are not applied: this is a relevant point because humans are simultaneously daily exposed to a wide variety of diverse substances which may have additive, synergistic,



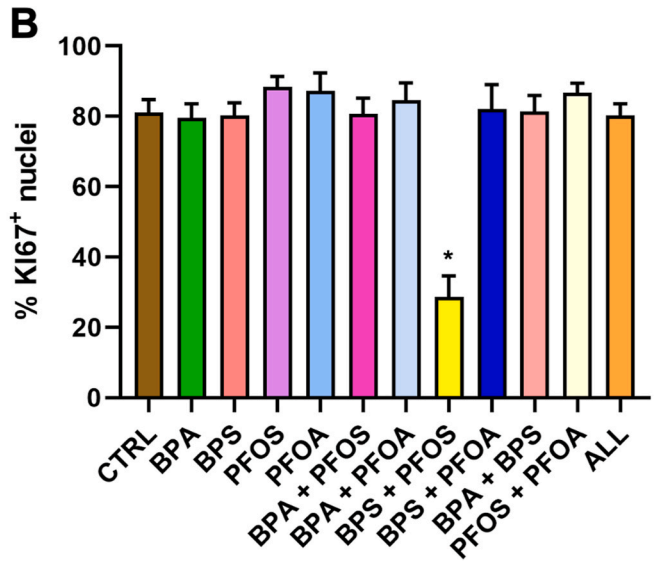


Fig. 3. Ki67 expression in hiPSCs exposed to PBs and/or PFs Immunocytochemical detection of Ki67 (green fluorescence) in hiPSC control cells and after 24 h exposure to vehicle (0.09% methanol) or to 0.1 µM of BPA, BPS, PFOS or PFOA alone or in combination, as reported. The nuclei were counterstained with DAPI (blue). Original magnification: 40×, scale bar 50 m. Images are representative of 3 independent experiments. Graph on the right indicates the % of the positive nuclei in the different experimental conditions. Data are expressed as mean ± SD ($n = 3$, *p < 0.05 vs Control).

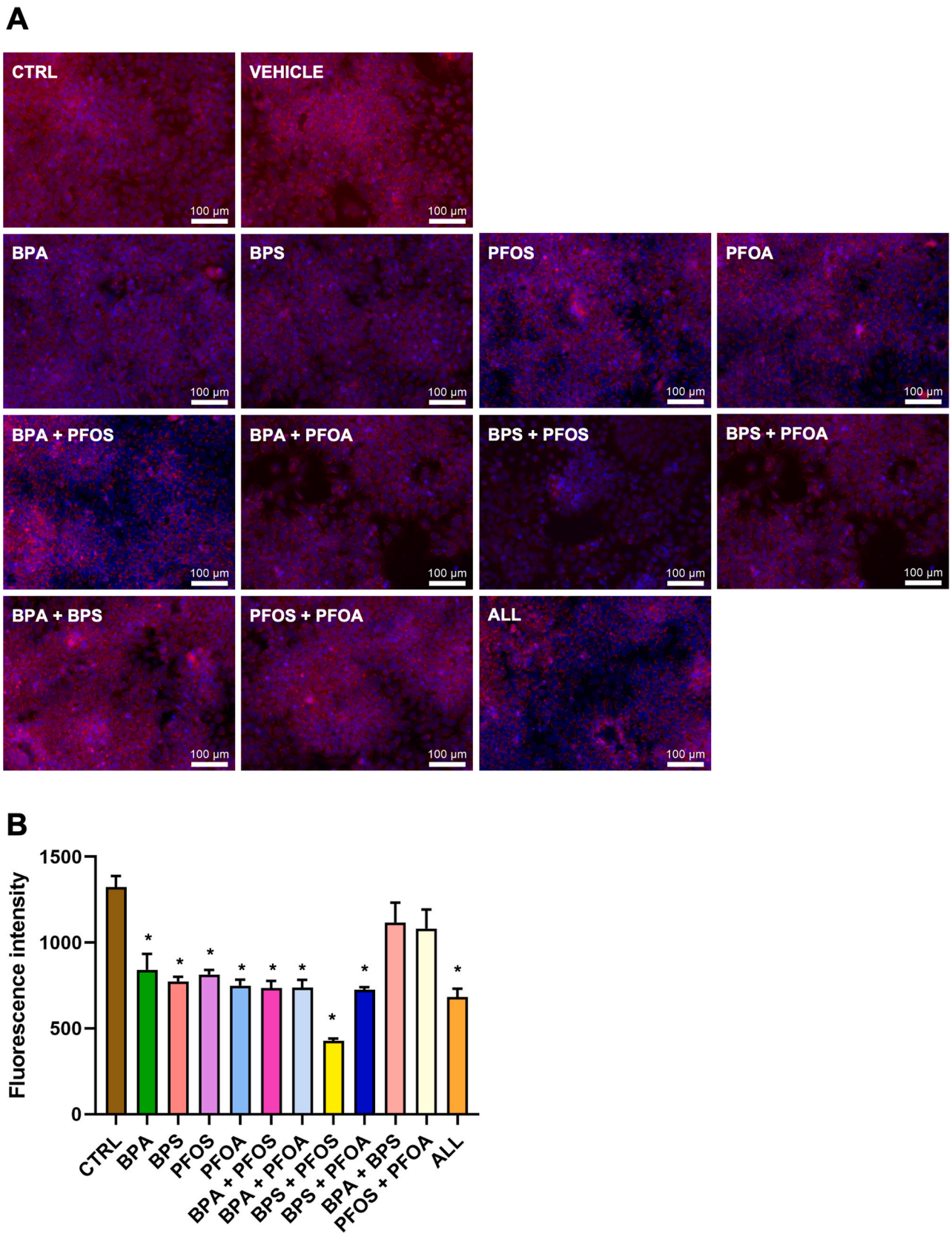


Fig. 4. Effects of exposure to BPs and/or PFs on hiPSCs mitochondrial health Immunocytochemical detection of MMP (red fluorescence) in Control cells and after 24 h exposure to vehicle (0.09% methanol) only or to 0.1 μM of BPA, BPS, PFOS or PFOA alone or in combination, as indicated. The nuclei were counterstained with DAPI (blue). Original magnification: 20×, scale bar 100 m. Images are representative of 3 independent experiments. Graph indicates the fluorescent intensity (absolute values) in the different experimental conditions. Data are expressed as mean ± SD ($n = 3$, *p < 0.05 vs Control).

Table 2
Effects of a 48 h exposure to the diverse pollutants on cell proliferative rate.

Pollutant (dose)	Impedance value (fold change vs. control)
BPA (0.1 μM)	0.95
BPS (0.1 μM)	0.94
PFOS (0.1 μM)	0.99
PFOA (0.1 μM)	1.01
BPA (0.1 μM) +PFOS (0.1 μM)	1.09
BPA (0.1 μM) +PFOA (0.1 μM)	0.93
BPS (0.1 μM) +PFOS (0.1 μM)	**0.27**
BPS (0.1 μM) +PFOA (0.1 μM)	1.01
BPA (0.1 μM) +BPS (0.1 μM)	**1.22**
PFOS (0.1 μM) +PFOA (0.1 μM)	1.02
BPA (0.1 μM) +BPS (0.1 μM) + PFOS (0.1 μM) +PFOA (0.1 μM)	**1.23**

Bold red or black values in bold represented statistically significant decreased and increased foldchange vs control, respectively ($p < 0.05$).

or negative biologic effects (Di Baldassarre et al., 2018b), (Kumar et al., 2020). Strengths of this study are that we used concentrations that resemble the xenobiotic levels detected in maternal and infant samples (Cairrao, 2022) (Jin et al., 2020)– (Rivera-Núñez et al., 2023) and that we explored the BPs and PFs effects as single chemicals or in combinations. The BPs and PFs toxicity has been tested on hiPSCs by evaluating the cell behavior in culture (Impedance), the response of the mitochondria, and the expression of highly characterizing genes. Our data confirm the difficulties to predict the effects of a cocktail of EDs: indeed, we evidenced that the combined exposure to molecules belonging to the two different classes studied did not induce, with some highly toxic exceptions (BPS + PFOS), an additive or synergic effect; interestingly, when two chemicals of the same class were paired (BPA + BPS or PFOS + PFOA), a negative interaction was observed in the analysis of both the MMP and gene expression; finally, the cocktail of all four pollutants studied generally exerted less severe effects than the single molecule. All these results highlight that it is very difficult to predict the "cocktail effect" of EDs, as some combinations are very toxic (such as BPS + PFOS), while in other cases the concomitant presence of two or more pollutants may mitigate the effect of the exposure, especially when cells are treated by molecules belonging to the same class: this can be due to a mechanism of competition for the same receptors or to receptor desensitization; anyway, the molecular mechanisms underlying the different responsiveness to the combined pollutants need more investigations.

Mitochondria are structurally complex organelles that provide energy to the cells and modulate cell signaling pathways associated with cell proliferation, differentiation, cell cycle progression, and apoptosis (Osellame et al., 2012). The integrity of MMP is essential for maintaining mitochondrial structure and function; therefore, its alteration is an indicator of mitochondrial dysfunction. Our data evidenced that BPs and PFs exerted an important mitotoxic activity in hiPSCs. The mitochondrial number, morphology, and distribution in hiPSCs are remarkably similar to those of embryonic cells, and the majority of cellular energy is expended to sustain the cell growth and at the same time to preserve their specific features of self-renewal and potency (Bukowiecki et al., 2014); accumulating evidence suggests that mitochondrial integrity is fundamental for stem cell viability and their proliferative and differentiative potential (Parker et al., 2009). Although the molecular mechanisms through which BPs and PFs induce mitotoxicity are far to be fully elucidated, previous data evidenced that PFs can accumulate inside the of eukaryotic membranes altering ion permeability and thus inducing changes in membrane potential (Qiao et al., 2019), (Kleszczyński and Składanowski, 2009).

The observation that BPs and PFs exposure is linked to unfavourable outcomes not only at birth but also later in adulthood (Napso et al., 2021), (Ghassabian et al., 2022) suggests that the fetal epigenome may be a critical target of these EDs, as epigenetic modifications in stem cells may influence both developmental process and susceptibility to disease later in life (Developmental Origins of Health and Disease). Among the epigenetic mechanisms, DNA methylation plays a critical role during early embryo development by regulating stimulation or repression of the development-related genes and timely establishing maternal and paternal imprints. It is strictly regulated by the opposite activities of DNMTs and TETs enzymes. We found that *DNMT1*, whose primary

Table 3
The table provides a summary of positive (+) or negative (−) modifications induced by the EDs, as single molecules or combined, proliferation rate, mitochondrial health, and gene expression of hiPSC.

		BPA	BPS	PFOS	PFOA	BPA + PFOS	BPA + PFOA	BPS + PFOS	BPS + PFOA	BPA + BPS	PFOS + PFOA	ALL
Proliferation Rate	Impedance							− −		+		+
	$Ki67^{+}$							− −				
Mitochondrial Health	MMP	−	−	−	−	−	−	− −	−			−
Pluripotency Markers	*NANOG*			+	+		+	+				
	OCT4			+	+	+		+				−
	SOX2								+			
	ESG1	+++	+	+++	+++	+++	+	+	++			
	KLF4	+	++	+	+	++	+	+	++		+	+
	REX1	− −	− −			− −	− −					− −
Germ cell Identifiers	*BLIMP1*	+										
	AP2G	−										
	PRDM14	−				−			−			− −
	STELLA	−	−			−	−					−
	FRAGILIS			− −			− −					
	OVOL1	− −		++	− −	− −			− −			
	PIWIL2	− −	−			− −	−		− −			−
Epigenetic Regulators	*DNMT1*		+									
	DNMT3A	+	+		+		− −	+				
	DNMT3B		+				− −			+		− −
	TET1		−						−			
	TET2	+	+			+						
	TET3	+	+	−	+	+	−	+				+

MMP: Mitochondria Membrane Potential. For gene expression, the entity of the modification was considered, using: three categories for increasing gene expression values (+= until 3-fold increase, ++ = between 3 and 5-fold increase, +++ = more than 5-fold increase), and two categories for decreasing gene expression values (− = until 0.5-fold decrease, − − = more the 0.5-fold decrease).

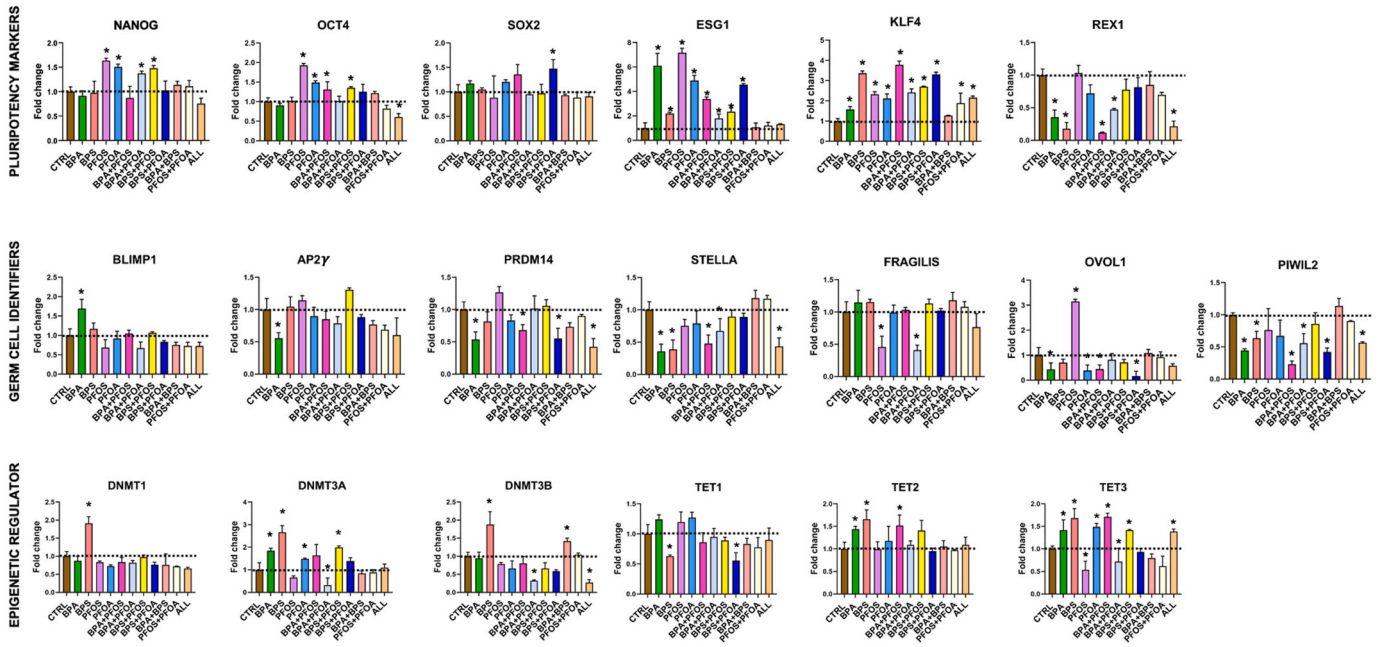


Fig. 5. Pluripotency, Germline, and epigenetic gene expression in hiPSCs upon BPs and/or PFs treatment. The gene expression of the stemness markers (*NANOG, OCT4, SOX2, ESG1, KLF4* and *REX1),* germinal lineage identifiers (*BLIMP1, AP2γ, PRDM14, STELLA, FRAGILIS, OVOL1* and *PIWIL2*), and epigenetic regulators (*DNMT1, DNTM3A, DNMT3B, TET1, TET2,* and *TET3*) were detected by qPCR in hiPSCs treated with BPs and/or PFs for 24 h, as indicated. The fold changes were determined from the $-\Delta\Delta$Ct values calculated using *18S* as a reference gene and normalized to untreated hiPSCs, as control condition (CTRL). The graphs show the mean ± SD of 3 independent experiments, $^{*}p < 0.05$ vs CTRL.

function is the maintenance methylation during the cell replication, was only scarcely affected by chemical exposure, while both *DNMT3A* and *DNMT3B*, which are essentially responsible for de novo methylation of the CpG islands, were diversly modulated, as BPs and PFOA, singularly administered, increased their expression, while BPA + PFOA or all four EDs together downregulated their transcripts. *TETs* were affected in a similar way by the treatments, being altered by BPs and PFs, alone or in different combinations. These modifications in the expression levels of enzymes responsible for the control of DNA methylation may have important consequences during the early stages of development. In fact, *DNMT3A* and *DNMT3B* display remarkable fluctuations from the Germinal Vesicles oocytes to blastocyst stage and keep on modifying in implanted embryos (Uysal et al., 2017), while TETs control the two demethylation waves that characterized the mammalian embryo development: the first follows the fertilization when both maternal and paternal genomes are globally demethylated in zygotes, the second occurs in PGCs, when TETs, especially TET1 and TET2, regulate a crucial set of germline reprogramming-responsive genes, involved in gamete generation and meiosis (Yang et al., 2020). Indeed, mice double knockout for Tet1 and Tet2 display reduced fertility and smaller ovaries in the case of females (Dawlaty et al., 2013). The evidence that BPs and PFs may interfere with epigenetic regulators such as DNMTs and TETs confirms the hypothesis that these EDs can alter fetal reprogramming, thus affecting the developmental processes, fertility, and susceptibility to disease later in life. It is worth noting that BPS, which has been introduced as an alternative in many commercial products available on the market as "BPA-free", has been found in human placenta at concentrations higher than that detected in the maternal serum, suggesting an accumulation of this ED in the perinatal tissues (Pan et al., 2020). The observation that BPS exposure deeply modifies the transcription of all DNMTs and TETs cast a dark shadow also on this chemical, evidencing that the replacement of BPA with BPS does not represent a healthier choice for human health.

In recent years, the incidence of infertility has gradually increased, becoming a global health problem, as WHO estimates that the 15% of the reproductive-age couple are affected by infertility, with a drastic decline in the number and quality of gametes representing the main causes (Bold and Swinburne, 2022). EDs first attracted public health attention because of reproductive anomalies in both wildlife and humans and now the recent advances in the understanding of infertility point out the involvement of environmental and lifestyle factors. BPA exposure has been associated with reproductive dysfunction in both men and women due to its ability to interact with estrogenic, androgenic, and thyroid hormone receptors (Matuszczak et al., 2019), and its substitute BPS has been shown to have the same hormone-disrupting abilities as BPA (Rochester e and BoldenBisphenol, 2015). The role of PFs is mainly derived from epidemiological and observational studies, that evidence a link between urinary PFs metabolites and impaired reproductive function, while antenatal exposure to PFs is associated with the prevalence of genital birth defects (Radke et al., 2018). Despite this growing evidence, a significant knowledge gap is how these BPs and PFs may affect reproductive function. In this study we highlighted for the first time that these EDs alter the network of molecules that control the complex processes of early development presiding over the pluripotency and the germline specification. During mammalian preimplantation development, a group of molecules, including *OCT4, NANOG, SOX2*, *ESG1* and *REX1,* finely orchestrates the pluripotency of the stem cells within the inner cell mass, from which both somatic and germline cell lineages will derive (Campolo et al., 2013) (Western et al., 2005). While the majority of epiblast cells undergo differentiation towards somatic cell lineages, PGCs, responsible for generating gametes, initiate a unique cellular program driven by the cooperation of the transcription factors BLIMP1, PRDM14 and AP2γ. These factors synergistically suppress the ongoing somatic differentiation and drive the re-expression of pluripotency and germ cell-specific genes accompanied by global epigenetic changes (Günesdogan et al., 2014). We found that in general BPs and PFs, alone or in combination, modify the biological characteristics of the hiPSCs by upregulating the pluripotency markers and downregulating the germline identifiers. The interference with these genes that are strictly and timely regulated during the pre- and post-implant phases strongly suggests the potential role of these chemicals in the increased trend of the infertility rate. Our results evidenced indeed that *BLIMP1* and *AP2γ* were

modified by exposure to BPA, while *PRDM14* was downregulated also by BPA + PFOS and BPS + PFOA, and deeply reduced when all the EDs were combined. The role of *PRDM14* in PGCs specification is critical as it upregulates germline-specific genes, assists BLIMP1-mediated repression of somatic transcripts, and initiates global epigenetic reprogramming (Yamaji et al., 2008). We also detected important modifications in the expression of genes that plays a critical role in supporting female (*STELLA* and *FRAGILIS*) and male (*OVOL1* and *PIWIL2*) gametogenesis. In particular, *STELLA* plays a significant role in oocytes and early embryonic development, modifies the higher-order chromatin structure to allow the expression of genes specifically expressed by female germ cells and its deficiency dramatically inhibits oocytes maturation inducing infertility (Hou et al., 2022); *Fragilis* is associated with the acquisition of germ cell competence by epiblast cell (Lange et al., 2003), and it is specifically expressed by oogonia stem cells in fetal gonads and by postnatal ovaries (Sheng et al., 2019). In our experiments, *STELLA* was affected by exposure to BPs in single, when BPA was combined with PFs and when all four EDs were pooled, while *FRAGILIS* was dramatically reduced by PFOS and by BPA + PFOA. These observations indicate that BPs and PFs exposure may alter female fertility by interfering with oogenesis. On the other side, *OVOL1* and *PIWIL2* were even more affected by the cell treatments, being dramatically reduced by BPA, alone or in combination with PFOS, and by BPS + PFOA. In mice, ablation of Ovol1 leads to a dramatic decrease in the production of spermatozoa, as germ cells are defective in progressing through the pachytene stage (Li et al., 2005). PIWIL2 is expressed in PGCs at embryonic stage and meiotic germ cells in adult animals. PIWIL2 is important for the maintenance and differentiation of spermatogonia and in Piwil2 mutant mice, spermatogenesis is disrupted at early prophase of meiotic division (Thomson and Lin, 2009). The evidence that BPs and PFs may downregulate genes critical for spermatogenesis reinforces the link between environmental contaminants and male infertility, indicating a possible mechanism through which these EDs may impact sperm health. It is also worth noting that among the pluripotency genes tested, *REX1* is the only one heavily downregulated by BFs, alone or in combination with PFs. This finding further worsens the possible EDs effects on the germline cells, as Rex1 mRNA is also expressed in spermatocytes actively undergoing meiosis and in dividing cells of the human testes and ovaries (Masui et al., 2008).

6. Conclusion

In conclusion, our results demonstrate that BPs and PFs impact stem cell integrity and support the hypothesis that these pollutants may impair reproduction by targeting the interplay among the multiple pluripotency regulators and the molecules expressed in PGCs and/or by gametes postnatally. Given that germline specification and gametogenesis are elaborate processes that entail also deep changes in the chromatin, such as DNA compaction and epigenetic remodeling, the combined effects of BPs and PFs on germline markers, pluripotency regulators and epigenetic regulators may result in quantitative and/or qualitative defects of gametes and consequent hypofertility or infertility. Moreover, BPs and PFs, modifying DNMTs and TETs, may target the fetal epigenome impacting the health outcome of the offspring not only at birth but also later in adulthood. All these data further highlight the need for wider awareness of the complexity of the EDs effects on human health and of the social and economic burden attributable to these compounds.

6.1. Study limitation

- BPs and PFs interfere with the androgen and estrogen receptors, and for this reason, they can induce possible sexual dimorphic effects. In this study, experiments were performed using hiPSCs line generated from Caucasian males. The possibility of a quantitative and/or qualitative diverse response due to sex specificities must be taken into account.
- In this study the "cocktail effect" was analyzed by pooling EDs at a single concentration, but in the "real life", humans are exposed to a combination of different chemicals at different concentrations: a deeper analysis of the EDs effects pooled at different concentrations is needed.
- Possible effect of exposures longer than 24 h were not evaluated for the gene expression and mitochondrial activity

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Ethics approval

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Consent to participate

Not applicable.

Consent for publication

All authors consent for publication.

Availability of data and material

Data are available on reasonable request from the corresponding author.

Code availability

Not applicable.

CRediT authorship contribution statement

Giulia Gaggi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Andrea Di Credico:** Data curation, Formal analysis, Investigation, Methodology. **Federica Barbagallo:** Conceptualization. **Barbara Ghinassi:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Angela Di Baldassarre:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Angela Di Baldassarre reports financial support was provided by Government of Italy Ministry of Education University and Research. Giulia Gaggi reports financial support was provided by European Union.

Data availability

Data will be made available on request.

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