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Cumulative and potential synergistic effects of seven different bisphenols on human granulosa cells *in vitro*?[☆]

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ABSTRACT

Bisphenol (BP) structural analogues of BPA are widely used. Previous studies showed similar effects of BPA and BPS on reproduction in several species including human. We hypothesised that the similar effects of several bisphenols (BPs) could accumulate in granulosa cells (GCs) and affects steroidogenesis. This study investigated the effects of seven BP analogues and their equimolar cocktail on human granulosa cells (hGC) and assessed BPA, BPS, BPF and BPAF level exposures in the follicular fluid of 277 women undergoing Assisted Reproductive Technology. The hGCs were recovered after women oocyte punctures and treated with the seven BP analogues (BPS, BPA, BPAF, BPF, BPAP, BPE and BPB) or their equimolar cocktail of 7×1.43 or 7×7.14 μM for each of the seven BPs, the sum of BPs reaching 10 (" $\sum\text{BPs } 10 \mu\text{M}$ "), or 50 μM (" $\sum\text{BPs } 50 \mu\text{M}$ "), respectively. Oestradiol and progesterone secretion, cell proliferation, viability and expression of steroidogenic enzymes were investigated. Progesterone secretion was decreased by 6 BPs 10 μM and the cocktail " $\sum\text{BPs } 10 \mu\text{M}$ ", (-17.8 to -41.3%) and by all seven BPs 50 μM and " $\sum\text{BPs } 50 \mu\text{M}$ " (-21.8 to -84.2%). Oestradiol secretion was decreased only by 50 μM BPAF and BPAP (-37.8% and -44% , respectively), with corresponding decreases in *CYP17A1* and *CYP19A1* gene expression. Cellular proliferation was decreased after treatment with 50 μM BPAF (-32.2%), BPAP (-29%), BPB (-24%) and the equimolar cocktail " $\sum\text{BPs } 50 \mu\text{M}$ " (-33.1%). BPB (50 μM) and the cocktail " $\sum\text{BPs } 50 \mu\text{M}$ " increased *HSD3B2* mRNA expression. At least one BP was detected in 64 of 277 (23.1%) women follicular fluids.

Similar effects of the seven BPs or their cocktail were observed on progesterone secretion and/or on cell proliferation, suggesting cumulative effects of BPs. Our results highlight the urge to consider all BPs simultaneously and to further investigate the potential additive or synergistic effects of several BPs.

1. Introduction

The human population has increasingly resorted to assisted reproductive technology (ART) due to a decline in fertility in both men and women (Inhorn and Patrizio, 2015). This observation raises the question of the impact of environmental factors on reproductive function, especially endocrine disruptors that could alter ovarian follicle development. Among the endocrine disruptors, bisphenols (BPs) are a family of aromatic organic compounds with two phenol groups (European-Food-Safety-Authority, 2015; Usman and Ahmad, 2016). Bisphenol A (BPA), is used as a plasticiser to produce polycarbonate plastics and epoxy resins, present in food containers, water pipes, baby bottles,

medical equipment (Chen et al., 2016) and in lab consumables (Togola et al., 2021). The main route of exposure is through the diet, due to the transfer of molecules present in food packaging to their contents, but it also occurs through dermal transmission and inhalation (Vandenberg et al., 2007; Vandenberg et al., 2010). BPA is, therefore, found in several human fluids and tissues, including urine, follicular fluid and blood (Calafat et al., 2005; Fernandez et al., 2007; Ikezuki et al., 2002). BPA is an oestrogen mimetic, it can bind to oestrogen receptors (ERs), such as ESR1 and ESR2 (Kuiper et al., 1998; Machtinger and Orvieto, 2014).

BPA exhibits adverse effects on human health; such as thyroid disruption, diabetes, cardiovascular diseases, obesity and deleterious effects on male and female reproductive functions (Lang et al., 2008;

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Peretz et al., 2014; Rochester, 2013). As BPA has been reported as an endocrine disruptor, it has been regulated and is now banned from the food industry in Canada, France and Belgium (European-Food-Safety-Authority, 2015; Usman and Ahmad, 2016). BPA is now replaced by other BPs that are structural analogues (Chen et al., 2016).

These molecules (i.e. BPA, BPS, BPAF, BPF, BPAP, BPE, BPB) are all used as polymers in the plastic industry to produce ordinary plastics, containers, coatings, thermal papers, plastic fibers and are therefore not only used in the food industry but also in the electronics, automobile, interior decoration, aerospace, and other industries (Chen et al., 2016; Loganathan et al., 2023; Neil et al., 2006; Rochester and Bolden, 2015). These seven BPs have also already been measured in human biological fluids, plastic consumables or cell culture and ART media (Gao et al., 2021; Togola et al., 2021; Zhan et al., 2023). Indeed, BPA and his analogues BPS, BPF, BPAF, BPAP, BPB were measured in human serum (0.071 ng/mL to 0.765 ng/mL and above 42 ng/mL for BPA) with a detection rate ranging from 72% to 100%, except 27% for BPB (Gao et al., 2021) and/or in urine samples with a detection rate 12%–93% in children, depending on the bisphenol analogues (Chen et al., 2018), and over 77% in adults (Zhan et al., 2023). Other studies also reported the presence of BPA, BPS, BPE, BPF, BPAF, BPAP, BPB in the environment (Chen et al., 2016) and/or in cell culture and ART media in concentrations ranging from 10 ng/L for the BPAF to 1693 ng/L for the BPS (Togola et al., 2021). The human population is therefore already exposed to these molecules. Among them, BPAF, BPF, and BPS are the most widely used (Wang et al., 2017).

One of the BP mechanisms explaining reproductive function disruption is the impairment reported on granulosa cell (GC) steroidogenesis, critical for sex steroid synthesis. However, several studies showed that the effects of BPs on GC steroidogenesis depend on the species, the BP analogue assessed and the concentration. Indeed, BPA and BPS inhibited progesterone secretion in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a; Grasselli et al., 2010), rat GC (Samardzija et al., 2018), ovine GC (Teteau et al., 2020) and human GC (Amar et al., 2020; Mansur et al., 2016). While the effect on progesterone secretion is constant, BPA and BPS could either stimulate, inhibit or have no impact on oestradiol secretion, depending on its concentration. In bovine GCs, 100 μ M BPS increased the secretion of oestradiol (Campen et al., 2018). Exposure to 0.087 μ M BPA (Mansur et al., 2016) or 50 μ M BPS (Amar et al., 2020) decreased oestradiol production in human GC (hGC). BPA and BPS (1 and 10 μ M) inhibited oestradiol secretion in porcine GC (Berni et al., 2019), whereas 0.1 μ M BPA stimulated oestradiol production (Grasselli et al., 2010). In another porcine GC study, BPA had no effect at any concentration on oestradiol secretion, whereas BPS decreased it from 1 μ M (Bujnakova Mlynarcikova and Scsukova, 2021a). BPS also impaired oocyte developmental competence in sheep (Desmarchais et al., 2020) and mice (Nourian et al., 2017).

Regarding the other less studied BPs, an absence of effect was reported for BPAF and BPF on oestradiol production in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a). BPAF decreased cell viability at 0.1 μ M in the COV434 human granulosa cell line (Bujnakova Mlynarcikova and Scsukova, 2021b) and at 10 μ M in KGN cells (Huang et al., 2020). In male, BPA (10 nM), BPS (1 μ M), BPB (0.1 μ M) and BPF (1 μ M) all impaired testicular steroidogenesis, while only BPA and BPS decreased sperm motility, sperm count and sperm viability (Bahelka et al., 2021; Jambor et al., 2021; Wisniewski et al., 2015).

We hypothesised that due to their structural homology, BPA analogues would have a deleterious effect on hGCs and a potential cumulative effect. The objective of this study was therefore to investigate the *in vitro* effects of 6 BPA analogues (BPS, BPAF, BPF, BPAP, BPE, BPB) and the reference BPA at 10 and 50 μ M and their equimolar cocktails “ Σ BPs 10 μ M” and “ Σ BPs 50 μ M”, the addition of the BPs at 1.43 or 7.14 μ M for each of the seven BPs, reaching 10 or 50 μ M respectively, on cell viability and proliferation, progesterone and oestradiol secretion and steroidogenic enzyme expression. These concentrations of 10 and 50 μ M were chosen as they were already reported for BPA and BPS to exhibit

effects on steroidogenesis in human and ovine GCs (Amar et al., 2020; Teteau et al., 2020; Teteau et al., 2023). In parallel, the environmental exposure to BPA, BPS, BPF and BPAF of 277 women undergoing ART protocol in France was measured in the follicular fluid.

2. Materials and methods

2.1. Chemicals and antibodies

BPS (CAS number: 80-09-1; purity: \geq 99%), BPA (CAS number: 80-05-7; purity: \geq 99%), BPF (CAS number: 620-92-B; purity: \geq 98%), BPAF (CAS number: 1478-61-1; purity: \geq 98%), BPE (CAS number: 2081-08-5; purity: \geq 98%), BPAP (CAS number: 1571-75-1; purity: \geq 98%) and BPB (CAS number: 77-40-7; purity: \geq 98%) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All other chemicals were obtained from Sigma-Aldrich unless otherwise stated in the text.

2.2. Bioethics

The hGCs were collected during oocyte retrieval from women undergoing IVF. This study was approved by the local review board of our university hospital and by the French Ministry of Research (DC-2014-2285), each patient had to sign previously an agreement form allowing the use of their GCs, which are otherwise discarded. All patients, except women with ovarian problems, were included in this study.

2.3. Follicular fluid sample collection and analysis

In this study, follicular fluid samples from 277 women undergoing ART procedure were collected and kept in glass tubes at -20° C until assays. Quantification without a hydrolysis step was performed on BPS glucuronide (BPSg), BPA glucuronide (BPAg), BPF glucuronide (BPFg) and BPAF glucuronide (BPAFg) by liquid chromatography-mass spectrometry with an Acquity U-HPLC device coupled to a Xevo-TQ triple quadrupole mass spectrometer (Waters, Saint-Quentin-en-Yvelines, France) operating in positive electrospray ionisation and MRM mode according to the method previously described (Gély et al., 2021). The limit of quantification (LOQ) was set at 0.05 ng/mL.

2.4. Isolation of human granulosa cells and *in vitro* cultures

The hGCs were collected from oocyte punctures on 277 women in ART in the service of Medicine and Biology of Reproduction in the CHRU of Tours, after ovarian stimulation treatment. After centrifugation (5 min, 400 g), follicular fluids were collected and stored at -20° C until BP analysis. The hGCs were collected as previously described (Amar et al., 2020). Briefly, cells were washed and resuspended with ACK (Ammonium Chloride 155 mM; Potassium Bicarbonate 10 mM; EDTA 0.10 mM) for 3 min at room temperature (RT) to lyse red blood cells. Then, the hGCs were washed, centrifuged (5 min, 400 g, RT) and resuspended in McCoy's 5 A medium, supplemented with L-glutamine 3 mM; 0.1% bovine serum albumin; 5 mL of penicillin/streptomycin (10000 UI/mL); 20 mM HEPES (1 M); 96 nM 4-androstene-11 β -ol-3,17-dione; bovine apo-transferrin (5 mg/L); 0.12 μ M selenium and 1.74 μ M insulin).

Cells were then purified using a 50% Percoll density gradient centrifugation (30 min, 700 g, RT). After another wash in medium and centrifugation (5 min, 400 g, RT), cells were stained with trypan blue and counted in the Thoma chamber. The hGCs were plated overnight in 96-well plates at 100.000 living cells per well and cultured at 37° C overnight before treatment.

2.5. Treatment

The hGCs were treated the following day with the seven BP analogues or their equimolar cocktail or the ethanol solvent at the

corresponding concentration. BPs were used at 10 μM when used alone or at 1.43 μM for each of the seven BPs to constitute the equimolar cocktail “ $\sum\text{BPs } 10 \mu\text{M}$ ”, in ethanol (0.01% or 171 mM), an ethanol control was also used. BPs were also used at 50 μM when used alone or at 7.14 μM for each of the seven BPs to constitute the equimolar cocktail “ $\sum\text{BPs } 50 \mu\text{M}$ ”, in ethanol (0.05% or 856 mM), an ethanol control was also used. The cells were cultured in a humid atmosphere containing 5% CO_2 in air at 37 °C for 48 h for steroidogenesis, viability, cell proliferation or 24 h for gene expression experiments. Supernatants were stored at -20 °C until analysis.

2.6. Cell viability

Cell viability was assessed after 48 h of treatment according to two complementary methods:

2.6.1. CCK8

Cell viability was first assessed with a CCK8 kit (Cell Counting Kit-8, 96992, Sigma-Aldrich). The measure highlights the indirect reduction of the tetrazolium salt WST-8 to WST-8 formazan by dehydrogenases from living cells. First, 10 μL of CCK8 solution were added to each well, and the plate was incubated (37 °C, 5% CO_2) for 3 h 30 min. Then, the absorbance was measured at 450 nm using a Thermo LabSystems plate reader (ThermoFisher Scientific, Ilkirch, France). The number of live cells is proportional to the measured optic density. Results are presented as a ratio to the ethanol controls of each experiment as mean \pm SEM for 5 independent experiments with at least duplicates.

2.6.2. LDH assay

The second method using the LDH Assay (Lactate Dehydrogenase Activity Assay, MAK066, Sigma-Aldrich, St. Louis, MO, USA) was performed on 50 μL of supernatant. The measure highlights the Nicotinamide adenine dinucleotide (NAD) to NADH reduction by the lactate dehydrogenase enzyme (released by dead cells in culture medium). Thus, the higher the LDH activity, the higher the cell death. Results are presented as a ratio to the ethanol controls of each experiment as mean \pm SEM for 6 independent experiments in duplicates.

2.7. Cell proliferation

After 48 h treatment with BrdU at 10 mM in the presence of BP analogues (BPA, BPS, BPAF, BPF, BPAP, BPE, BPB) or their equimolar cocktail “ $\sum\text{BPs } 10 \mu\text{M}$ ” and “ $\sum\text{BPs } 50 \mu\text{M}$ ”, culture supernatant was removed and cell proliferation was measured with an enzyme-linked immunosorbent assay (cell proliferation ELISA, BrdU [colourimetric], Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a Thermo LabSystems plate reader and Ascent Software for Multiskan hardware. Cell proliferation was normalised to the control condition of each culture. Results are presented as a ratio to the ethanol controls of each experiment as mean \pm SEM for 5 independent experiments with at least duplicates.

2.8. Steroid hormone assays

Culture supernatants were collected after 48 h treatment and stored at -20 °C until oestradiol and progesterone measurement. First, 50 μL of lysis buffer (150 mM NaCl, 1 mM Tris, 1 mM EDTA, 2 mM EGTA, 2 mM Na_3PO_4 , 10 mM NaF, 12 mM NaH_2PO_4 , 0.5% (v/v) NP40 and 1% (v/v) Triton X 100) were added in each well to enable the normalisation of hormone concentration with the protein concentration of the corresponding well. Cell samples were stored at -20 °C until assay.

2.8.1. Progesterone assay

The measure of progesterone concentration was carried out by competitive enzyme immunoassay, according to the protocol previously

described (Canepa et al., 2008). The absorbance was measured at 405 nm with a Sunrise-basic plate reader (TECAN Life Sciences, Switzerland) and Magellan software. For progesterone concentrations that ranged from 0.25 to 32 ng/mL, the intra-assay coefficient of variation (CV) was <7%. The results, expressed in nanograms of progesterone per microgram protein, were then normalised to the control condition of each experiment and presented as mean \pm SEM of 15 experiments with three replicates per condition.

2.8.2. Oestradiol assay

The oestradiol concentration was measured using an enzyme immunoassay (DIASource, E2-EASIA-kit, Louvain-La-Neuve, Belgium), according to the manufacturer's instructions. Briefly, 50 μL of spent medium was used for the assay; the competition between unlabelled oestradiol and labelled oestradiol (respectively present in the culture media and provided by the kit) lasted 2-h at 4 °C. The interassay CVs averaged 17% for oestradiol concentration, which ranged from 1.56 to 50 pg/mL. The results, expressed in nanogram oestradiol per picogram protein, were then normalised to the control condition of each experiment and presented as mean \pm SEM of 13 experiments with two replicates per condition.

2.9. Protein extraction and quantification

After supernatant removal and the addition of lysis buffer to the cells (150 mM NaCl, 1 mM Tris, 1 mM EDTA, 2 mM ethylene glycol tetraacetic acid [EGTA], 2 mM Na_3VO_4 , 10 mM NaF, 12 mM NaH_2PO_4 , 0.5% NP40 (v/v), 1% Triton X-100 [v/v]), proteins were extracted on ice. The recovered lysates were then centrifuged for 25 min at 4 °C, at 16.000 \times g, and the supernatant protein concentration was measured using the BCAssay Protein Quantification colourimetric kit (Interchim, Montluçon, France), according to the manufacturer's recommendations. Absorbance was measured at 550 nm using a Thermo LabSystems plate reader and Ascent Software for Multiskan equipment.

2.10. Gene expression analyses

Six independent experiments collected after 24 h treatment in the presence or absence of the seven BP analogues or their equimolar cocktail “ $\sum\text{BP } 10 \mu\text{M}$ ” or “ $\sum\text{BP } 50 \mu\text{M}$ ” were used for transcriptomic analysis by real-time quantitative polymerase chain reaction (qPCR). Briefly, total RNA was extracted from hGCs using the RNA Nucleospin (Macherey Nagel, Düren, Germany), following the manufacturer's instructions. Subsequently, the RNA concentration was determined using Qubit plus kit (ThermoFisher Scientific, Eugene, Oregon, USA). Reverse transcription (RT) was performed on 150 ng total RNA extracted from hGCs using the Maxima First Strand cDNA Synthesis kit (ThermoFisher Scientific, Vilnius, Lithuania), according to the manufacturer's recommendations. qPCR reactions were performed as previously described (Amar et al., 2020) for androgen receptor (AR), oestrogen receptor 1, 2 (ESR1, ESR2), the cholesterol transporter (StAR) and steroidogenic enzymes involved in the progesterone synthesis (CYP11A, the enzyme transforming cholesterol into pregnenolone and HSD3B1, HSD3B2, the enzymes transforming pregnenolone into progesterone), androgen synthesis (CYP17A1) and oestradiol synthesis (CYP19A1 or aromatase). Apoptotic genes were also tested (BAX, Bcl 2) and five candidate genes highlighted in a previous RNAseq study characterising gene changes after BPA and BPS treatment in ovine GC (JUNH, KLF9, KLF10, LIF, TXNIP) (Téteau et al., 2023).

The geometric mean of two housekeeping genes (Ribosomal protein L19 [RPL19] and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) was used to normalise gene expression. The relative amounts of gene transcripts (R) were calculated according to the equation:

$$R = \frac{E^{-Ct_{gene}}}{(\text{geometric mean } (E^{-Ct_{GAPDH}}, E^{-Ct_{RPL19}}))}$$

where E is the primer efficiency (Table 1), and Ct is the cycle threshold.

2.11. Statistical analysis

Statistical analyses were performed with R version 4.2.1 software, using the R Commander package (R Core Team, 2015). Shapiro and Student's t tests were performed on age, body weight, height, BMI, number of cigarettes and number of oocytes punctured between women with detectable vs undetectable BP in their FF samples. Chi 2 analysis was also performed on the % of smoker. For steroidogenesis, cell proliferation, viability and gene expression, nonparametric analysis of variance (ANOVA) by permutation (lmpcr package) was performed due to a non-normal distribution (Shapiro test) and non-homogeneous variances (Levene test). Tukey's post hoc test (nparcomp package) was used to determine differences between groups. A p-value ≤ 0.05 indicated a significant difference, and $0.05 < p \leq 0.10$ indicated a tendency.

3. Results

3.1. Female follicular fluid BPA, S, F, AF glucuronide assays

Follicular fluid samples from 277 women were collected and assessed for glucuronide forms of BPA, BPS, BPF and BPAF (BPAg, BPSg, BPFg, and BPAFg, respectively) (Fig. 1 and Table 2). The average age of the population of women was 33.0 ± 0.3 years, the average BMI was 23.9 ± 0.6 with a mean weight of 64.5 kg and a mean height of 164 cm. In this cohort, 47 women were smokers (17%) and smoked in average 4 cigarettes per day. At least, one BP was detected in 64 of the 277 follicular fluid samples tested (i.e. 23.1% detection rate: 36 women for BPSg (13%), 33 for BPAg (11.9%) and 4 for BPFg (1.4%). The mean concentration was 0.483 ± 0.112 nM for BPSg, 0.184 ± 0.052 nM for BPAg, 0.347 ± 0.152 nM for BPFg. BPAFg was not detected among these 277 samples. A multi-exposure with BPA and BPS was observed for 3% of the patients (n = 9). An average of 10 oocytes were punctured for these women. None of these parameters showed a significant difference between women with detectable vs undetectable BP levels in their FF sample.

Table 1

Primer sequences.

Abbrev.	Name	Forward (5' à 3')	Reverse (5' à 3')	Size (bp)	E (%)
AR	Androgen receptor	CTCTGGTGGTTCCCTCTCTG	AGCATCCAAGTGGCTTATGG	165	103
BAX	BCL2 associated X	TCTGACGGCAACTTCAACTG	TTGAGGAGTCTACCCAACC	188	95.1
BCL2	B-cell lymphoma 2	CACCTGTGGTCCACCTGAC	ACGCTCTCCACACACATGAC	217	98.9
CYP11A1	Cytochrome P450 family 11 subfamily A member 1	TGGCTGAGCAAAGACAAGAA	AGGTGAAGGAGATGGGCTTT	214	98.9
CYP17A1	Cytochrome P450 family 17 subfamily A member 1	TGAGTTTCTGTGGACAAGG	TCCGAAGGGCAAATAGCTTA	163	91.3
CYP19A1	Cytochrome P450 family 19 subfamily A member 1	CCAGTGA AAAAGGGGACAAA	CCATGGCGATGTACTTTCTT	172	91.4
ESR1	Oestrogen receptor 1	TCCAACCTGCATTTCTTTTC	TTGGAACATGGCAGCATTTA	201	109.6
ESR2	Oestrogen receptor 2	GATGCTTTGGTTTGGGTGAT	ATCGTTGCTTCAGGCAAAAG	175	109
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GTCAGTGGTGGACCTGACCT	TGCTGTAGCCAAATTCGTTG	245	101
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	AGAGGCCTGTGTCCAAGCTA	CCAGAGGCTCTTCTTCATGG	116	102
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	ATCCACACCCGCTGTATCAT	TTTCCAGAGGCTCTTCTTCG	208	91.2
KLF9	KLF transcription factor 9	ACAGTGGCTGTGGAAAAGTC	AACTGCTTTTCCCAAGTGTG	169	103
KLF10	KLF transcription factor 10	CATCTGTAGCCACCCAGGAT	CATGCTTGGTCAAATGGTCA	236	90.7
JUNH	Jun proto-oncogene	CGAAAAAGGAAGCTGGAGAG	TGAGTTGGCACCACCTGTTA	160	90.2
LIF	LIF interleukin 6 family cytokine	CTGTTGGTTTGCACCTGGAA	GCCACATAGCTTGTCCAGGT	216	102
RPL19	Ribosomal protein L19	CATGGAACACATCCACAAGC	TTGGTCTCTTCTCCTTGGAT	171	92.5
STAR	Steroidogenic acute regulatory protein	CCTGAGCAGAAGGGTGTGCAT	AGGACCTGGTTGATGATGCT	151	96.6
TXNIP	Thioredoxin interacting protein	CCTGGTAATTGGCAGCAGAT	CTTGAGAGCCATCCATGTCA	200	90.3

3.2. Cell viability

No effect on cell viability was reported at 10 μ M in any condition after 48 h treatment, either with CCK8 or LDH activity assays compared to the control (Figs. 2A and 1C). No difference was found with LDH activity at 50 μ M in any condition after 48 h (Fig. 2B). In contrast, a significant decrease in cell viability was observed with 50 μ M BPAF (-51.8% , $p < 0.0001$) and 50 μ M BPAP (-72.6% , $p < 0.0001$) compared to the control with the CCK8 assay (Fig. 2D). To further investigate the toxicity of these two molecules, a CCK8 assay was also performed after 24 h treatment with BPAF and BPAP. No effect on cell viability was reported compared to the control for this shorter time exposure (Supplementary Fig. 1).

3.3. Cell proliferation

No difference in cell proliferation was measured after BrdU incorporation of all 10 μ M conditions compared to the control (Fig. 3 and Supplementary Fig. 2). On the contrary, at 50 μ M, a significant decrease in cell proliferation was observed for BPAF (-32.2% , $p < 0.0001$), BPAP (-29% , $p = 0.037$), BPB (-24% , $p < 0.0001$) and the equimolar cocktail " \sum BP 50 μ M" of the seven BPs (-33.1% , $p < 0.0001$). Moreover, the cocktail " \sum BP 50 μ M" condition is below the dotted line representing the average effect of the seven individual BPs.

3.4. Oestradiol secretion

After 48 h of treatment, no effect on hGCs oestradiol secretion was observed at 10 μ M in any condition (Fig. 4A and Supplementary Fig. 3). The 50 μ M BPAF treatment decreased oestradiol secretion by 37.8% ($p = 0.021$) and 50 μ M BPAP decreased oestradiol secretion by 44% ($p = 0.0008$) compared to the control. In contrast, the BPB treatment showed a significant 65% oestradiol increase ($p = 0.050$) compared to the control (Fig. 4B). To confirm the effect of BPAF and BPAP on oestradiol secretion in a condition that does not alter cell viability, oestradiol secretion was also measured for these two molecules after 24 h of treatment. However, no effect of BPAF and BPAP at 50 μ M after 24 h treatment was found (Supplementary Fig. 1).

3.5. Progesterone secretion

After 48 h of treatment, a significant decrease in progesterone secretion was observed for all the conditions and concentrations tested, except for 10 μ M BPF (Fig. 4C and D). A significant decrease in

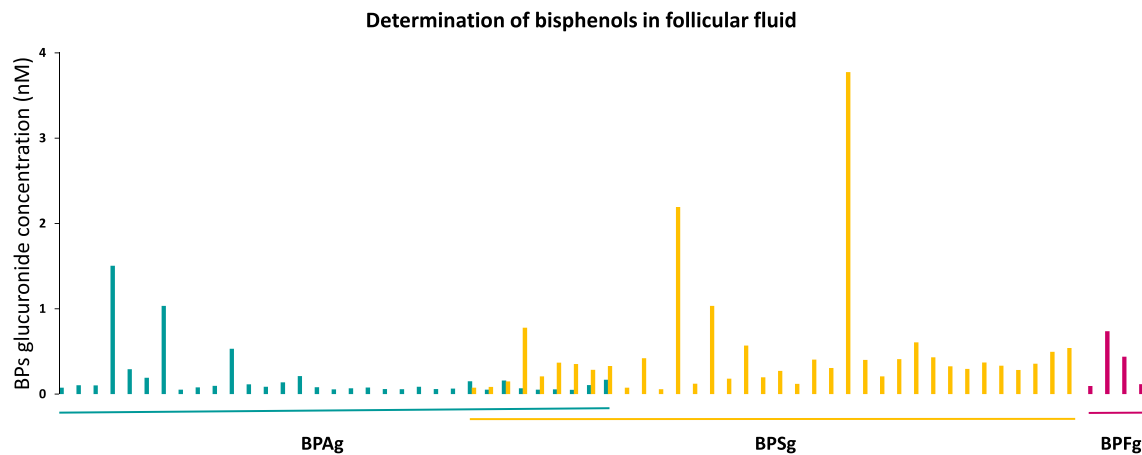


Fig. 1. Follicular fluid exposure to Bisphenol A, S, F and AF of women undergoing ART protocol. Follicular fluids were collected from 277 women, in Tours, France. BPAg (green bar), BPSg (yellow bar), BPFg (red bar) and BPAFg were measured using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC MS/MS). The histogram represents the bisphenol glucuronide concentrations in ng/mL of the 64 women samples (among 277 follicular fluid samples tested) where bisphenols were detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Characteristics of the study population.

	total women follicular fluid samples (n = 277)	Detectable BP in women FF samples (n = 64)	Undetectable BP in women FF samples (n = 213)	p-value (detectable vs undetectable)
BPAg detection rate	11.9%			
BPAg mean of exposure nM (min - max)		0.184 (0.05–1.505)		
BPAg mean of exposure ng/mL (min - max)		0.075 (0.020–0.609)		
BPSg detection rate	13%			
BPSg mean of exposure nM (min - max)		0.483 (0.057–3.774)		
BPSg mean of exposure ng/mL (min - max)		0.212 (0.025–1.654)		
BPFg detection rate	1.4%			
BPFg mean of exposure nM (min - max)		0.347 (0.096–0.737)		
BPFg mean of exposure ng/mL (min - max)		0.130 (0.036–0.277)		
BPAFg detection rate	0			
BPAFg mean of exposure nM (min - max)		–		
BPAFg mean of exposure ng/mL (min - max)		–		
BP glucuronide detection rate	23.1%			
BP glucuronide mean of exposure nM		0.389		
BP glucuronide mean of exposure ng/mL		0.166		
Age mean (min - max)	33.04 (17–43)	32.63 (18–41)	33.2 (17–43)	ns
body weight mean (min - max)	64.5 kg (40–123)	64.4 kg (44–107)	64.6 kg (40–123)	ns
Height mean (min - max)	164.3 cm (150–180)	163 cm (150–180)	164.7 cm (150–180)	ns
Body mass index mean (min - max)	23.9 (15.6–40.6)	24.2 (16.4–38)	23.8 (15.6–40.6)	ns
% of smokers	17	17.2	16.9	ns
mean number of cigarettes (min - max)	4.0 (1–15)	5.2 (1–15)	3.6 (1–15)	ns
mean number of punctured oocytes (min - max)	10.2 (0–31)	10.6 (1–31)	10.1 (0–30)	ns

BP: bisphenol; FF: follicular fluid; BPAg: bisphenol A glucuronide; BPSg: bisphenol S glucuronide; BPFg: bisphenol F glucuronide; BPAFg: bisphenol AF glucuronide; ns: not significant.

progesterone secretion was observed for 10 μM and 50 μM BPS (−17.8%, p = 0.049 and −51%, p < 0.0001, respectively), 10 μM and 50 μM BPA (−36.3%, p < 0.0001 and −47.6% p < 0.0001, respectively), 10 μM and 50 μM BPAF (−28%, p < 0.0001 and −84.2%, p < 0.0001, respectively), 50 μM BPF (−21.8%, p = 0.0004), 10 μM and 50 μM BPAP (−19.7%, p = 0.010 and −69.2%, p < 0.0001, respectively), 10 μM and 50 μM BPE (−41.3%, p < 0.0001 and −50.4%, p < 0.0001, respectively), 10 μM and 50 μM BPB (−25.8%, p < 0.0001 and −37.8%, p < 0.0001, respectively) and the equimolar cocktail “∑BP 10 μM” and “∑BP 50 μM” (−40.6%, p < 0.0001 and −48.7%, p < 0.0001, respectively). The cocktail “∑BP 10

μM” condition is below the dotted line representing the average effect of the seven individual BPs, while the “∑BP 50 μM” is similar to the dotted line. There was a dose effect for BPA, BPE, BPB and the seven-BP equimolar cocktail on progesterone secretion (Supplementary Table 1, Supplementary Fig. 4).

3.6. Gene expression analysis

The mRNA expression of 16 genes (Supplementary Tables 2 and 3), notably those of steroid and hormone receptors (AR, ESR1 and ESR2),

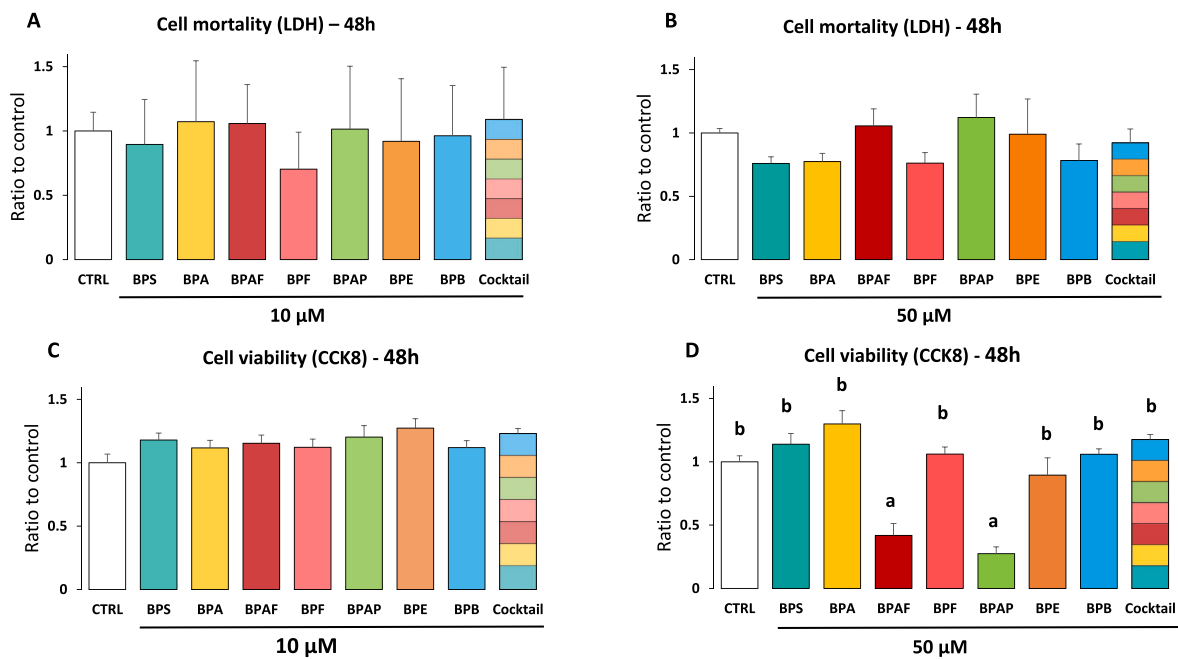


Fig. 2. Effects of seven bisphenols or their equimolar cocktail “ \sum BP 10 μ M” and “ \sum BP 50 μ M” on cell viability. Human GC underwent a 48 h culture in the presence or absence of BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or the cocktail (A and C: 10 μ M or B and D: 50 μ M). Regarding the equimolar cocktail, the addition of the seven bisphenols at 7×1.43 or 7×7.14 μ M reach 10 and 50 μ M, respectively. Bars with different superscripts indicate a significant difference (ANOVA, $p \leq 0.05$). (A and B) LDH activity was assessed in the culture supernatant and is inversely proportional to the optical density measured and cell viability. Results are presented as a ratio to the ethanol controls of each experiment as mean \pm standard error of the mean (SEM) for 6 independent experiments with duplicates. (C and D) hGC viability was assessed by CCK8 assay, and the number of live cells is proportional to the measured optical density; results are presented as a ratio to the ethanol controls of each experiment as mean \pm SEM for 5 independent experiments with at least duplicates.

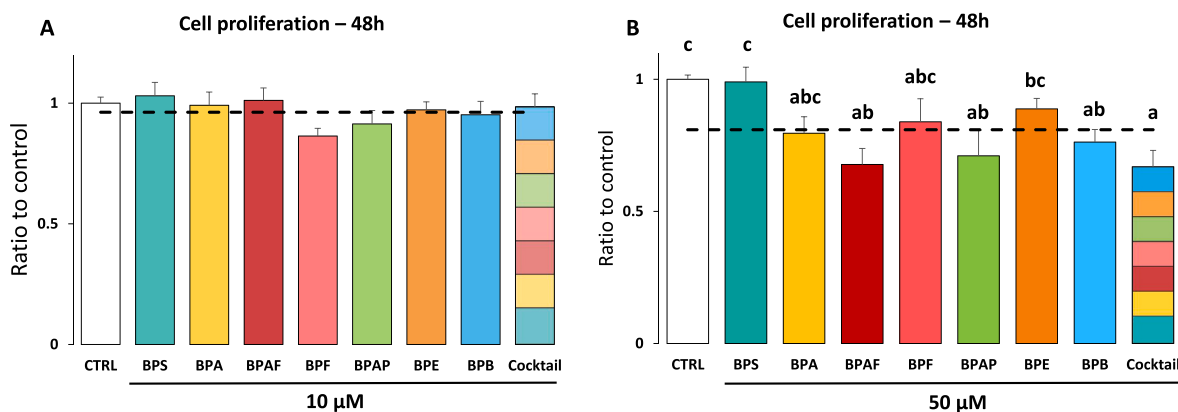


Fig. 3. Effect of seven bisphenols or their equimolar cocktails “ \sum BP 10 μ M” and “ \sum BP 50 μ M” on hGC cell proliferation. Human GC underwent 48 h culture with 10 μ M bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU), in the presence or absence of BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or the equimolar cocktail (A: 10 μ M or B: 50 μ M). Regarding the equimolar cocktail, the addition of the seven bisphenols at 7×1.43 or 7×7.14 μ M reach 10 and 50 μ M, respectively. The cell proliferation was normalised to the control condition of each culture. The dotted black line defines the average effect of the seven bisphenols. The results are expressed as the mean \pm SEM of five independent experiments with at least duplicates. Bars with different superscripts indicate a significant difference ($p \leq 0.05$).

genes involved in steroidogenesis (*StAR*, *CYP11A1*, *CYP17A1*, *CYP19A1*, *HSD3B1* and *HSD3B2*) and apoptotic factor genes (*BAX* and *BCL2*), was measured in hGCs using qPCR after 24 h in the presence or absence of the seven BP analogues or their equimolar cocktail (“ \sum BP 10 μ M” and “ \sum BP 50 μ M”). No significant difference was found for any gene at 10 μ M compared to the control (Supplementary Table 2). At 50 μ M, a significant decrease in *CYP17A1* and *CYP19A1* mRNA expression was observed for both BPAF and BPAP compared to the control. The BPAF treatment led to a significant 8-fold decrease in *CYP17A1* and *CYP19A1* expression ($p = 0.0002$ and $p = 0.004$, respectively) compared to the control (Fig. 4 and Supplementary Table 3). The BPAP treatment led to significant 15-fold and 8-fold decreases in *CYP17A1* and *CYP19A1*

expression ($p < 0.0001$ and $p = 0.003$), respectively, compared to the control. A significant increase in *HSD3B2* mRNA expression was also observed after the 50 μ M BPB treatment (1.6-fold, $p = 0.04$) and after cocktail “ \sum BP 50 μ M” treatment (2-fold, $p = 0.003$) compared to the control. No other BPs had any effect on the other analysed genes compared to the control (Fig. 5).

4. Discussion

For the first time, the effects of several BPs and their equimolar cocktail on hGC functions were studied. All the BPs assessed inhibited progesterone secretion except for BPF at 10 μ M. Even though each

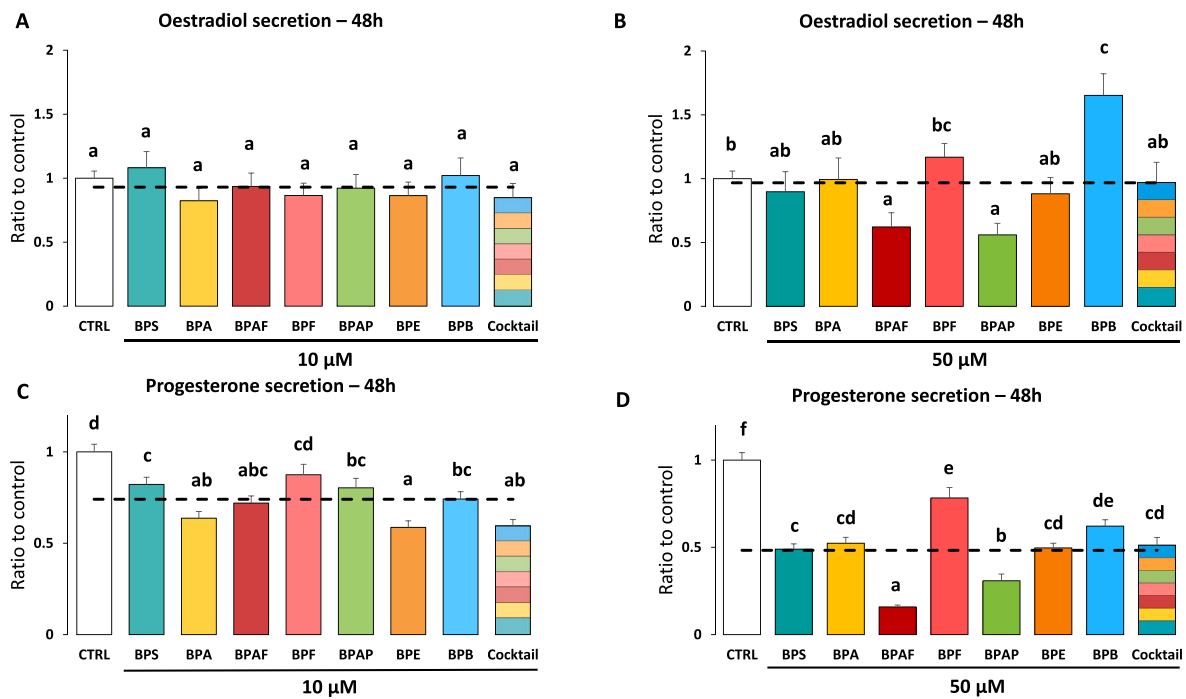


Fig. 4. Effect of seven different bisphenols or their equimolar cocktails on oestradiol and progesterone secretion. Human GC underwent 48 h culture in presence or absence of BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or their equimolar cocktails “∑BP 10 μM” and “∑BP 50 μM” at 10 (A and C) and 50 μM (B and D). Regarding the equimolar cocktail, the sum of the seven bisphenols at 7×1.43 or 7×7.14 μM reach 10 and 50 μM, respectively. (A and B) The oestradiol concentration of the culture medium was assessed after 48 h treatment by enzyme-linked immunoassay (ELISA). The results were normalised to the control condition of each experiment. Data are expressed as mean ± SEM in pg oestradiol per μg protein (13 independent experiments with two replicates per condition). (C and D) The progesterone concentration was measured in culture media, and its value was normalised by the protein concentration in each well. Data are expressed as mean ± SEM in ng progesterone per μg protein (15 independent experiments with 3 replicates per condition, except for the cocktail = 8 experiments with 3 replicates). The dotted black line represents the average effect of the seven individual bisphenols. Bars with different superscripts indicate a significant difference ($p \leq 0.05$).

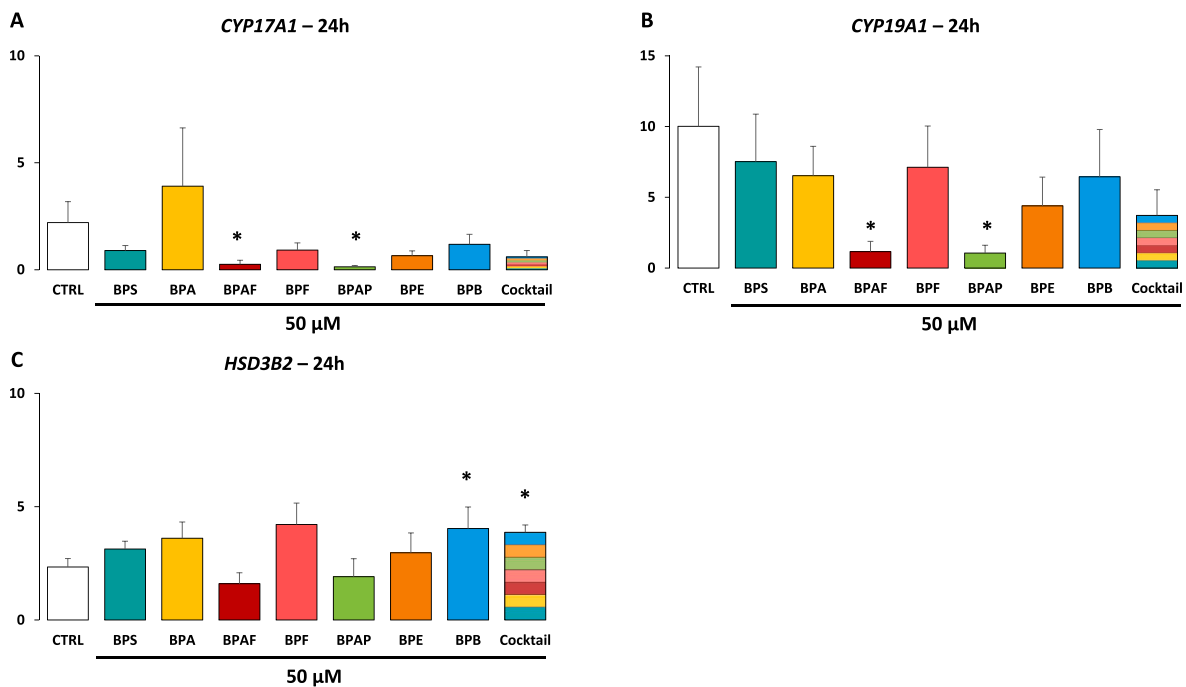


Fig. 5. Effects of seven different bisphenols or their equimolar cocktail “∑BP 50 μM” on transcriptional gene expression in hGCs. mRNA expression of three steroidogenic enzymes (*CYP17A1*, A.; *CYP19A1*, B and *HSD3B2*, C) was assessed in hGCs after 24 h of culture in complemented serum-free McCoy’s 5 A media in the presence or absence of seven bisphenols (BPS, BPA, BPAF, BPF, BPAP, BPE, BPB) or their equimolar cocktail “∑BP 50 μM”. Regarding the equimolar cocktail, the addition of the seven bisphenols at 7×7.14 μM reach 50 μM. Total mRNA was extracted from hGCs and reverse transcribed, and a real-time polymerase chain reaction (qPCR) was performed. The geometric mean of two housekeeping genes (*GAPDH* and *RPL19*) was used to normalise gene expression. The results are expressed as mean ± SEM of six independent cultures. * indicates a significant difference with the control condition ($p \leq 0.05$).

molecule was 7-fold less concentrated in the cocktail, the effect of the equimolar cocktail was similar to that of the individual molecules, suggesting a cumulative effect for the seven BPs assessed. Similarly, the effect of the cocktail “ \sum BP 50 μ M”, with seven BP at 7.14 μ M each, decreased cellular proliferation, while no effects was reported when each BP alone was at 10 μ M. Two BPs, BPAF and BPAP, decreased cell viability and might therefore be even more toxic compared to other BPs. Among the 277 women follicular fluid samples assessed for BP content, two different BPs were simultaneously detected in some women, thereby justifying the study of the combined effects of BPs.

1. Women exposure to bisphenols

BPs were detected in 23% of 277 women follicular fluids. The most common BP detected in follicular fluid was BPS, even though the number of women exhibiting BPA or BPS was almost the same. This could be explained because even if the BPA had been banned (European-Food-Safety-Authority, 2015; Usman and Ahmad, 2016), the regulation is not entirely applied in France. Moreover, dietary exposure to BPA has been estimated to be the most important route (Vandenberg et al., 2007), but other exposures, such as transdermal or inhalation exposure, could also occur. The frequency of BPS exposure in follicular fluid is close to BPA exposure. Comparatively, BPA, BPS, BPAF and BPF were detected in more than 72% of the 353 human serum from people working in a dense industrial area in China at an average concentration of 42.1 ng/mL, 0.07 ng/mL, 0.77 ng/mL and 0.19 ng/mL, respectively (Gao et al., 2021). BPA, BPS and BPAF were also measured in another Chinese study with more than 77% detection rate in the 733 human urine samples at an average concentration of 1.14, 0.12 and 0.09 ng/g creatinine (Zhan et al., 2023). The great difference in detection rate might occur because of both the biological fluid, levels that could be higher in serum and urine compared to follicular fluid, and the difference between population and spending habits (French population versus Chinese population). Whereas BPS seemed to be the first substitute for BPA in France, it does not seem to be the case in China where BPAF seemed to be second to BPA in biological fluids.

BP measurement in human follicular fluid is performed after 16 h of fasting. Because the BP half-life is quite short (around 6 h) (Khmiri et al., 2020), the percentage of the population exposed to BP is likely underestimated, especially because the concentrations measured in follicular fluid are close to the limit of detection of the method. However, an Italian team assessed BPA levels in ART patients under similar conditions (collection of serum and follicular fluid after overnight fasting) and found a similar detection rate of BPA in the follicular fluid as ours (28.7%) in 122 women at a range of 1.25–1.90 ng/mL and a higher detection rate of 52.4% in the serum of these same patients (Paoli et al., 2020). In Russia, another study based on 292 patients found a BPA exposure level of 16.8% in follicular fluid samples with a mean of 0.2–0.4 ng/mL (up to 56 ng/mL), while BPA was detected in 92.3% of blood samples of the same patients (Syrkasheva et al., 2021). In our study, three different BPs were detected in the follicular fluid: BPA, BPS and BPF. On the contrary, BPAF was not detected in any of the 277 samples. In addition, several patients showed simultaneous exposure to both BPA and BPS, as it is reported in other Chinese's studies (Gao et al., 2021; Zhan et al., 2023). This result highlighted the importance of considering simultaneously the several members of a family of molecules that exhibit similar effects.

2. Similar effects of several bisphenols on progesterone secretion

In this study, similar effects of several BPs and their cocktail were measured. Indeed, all seven BPs and their combination decreased hGC progesterone secretion at both concentrations. These results are consistent with the literature. BPA and BPS were already reported to significantly reduce progesterone secretion in hGCs (Amar et al., 2020; Mansur et al., 2016) or in a human KGN granulosa cell line (Shi et al.,

2021). Data from animal models also support this result. Indeed, BPA exposure decreased progesterone secretion in ewe GC (Teteau et al., 2020), porcine GC (Grasselli et al., 2010) and rat GC (Samardzija et al., 2018). BPA exposure also decreased progesterone secretion in bovine thecal cells (Tyner et al., 2022). Among BPA analogues, BPS decreased progesterone secretion in ewe GC (Teteau et al., 2020) and cumulus cells (Desmarchais et al., 2020) but not in bovine GC (Campen et al., 2018). BPF and BPAF decreased progesterone secretion in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a), and BPC decreased progesterone secretion in bovine thecal cells (Tyner et al., 2022). However, controversial effects are highlighted in swine and rats, where opposite effects on progesterone secretion were reported depending on the concentration, i.e. 1 μ M BPA treatment increased progesterone synthesis whereas 100 μ M BPA treatment decreased it (Mlynarcikova et al., 2005; Zhou et al., 2008). Moreover, in bovine thecal cells, BPF increased progesterone secretion (Tyner et al., 2022). Effects of BPE, BPAP and BPB were not reported to date in granulosa cells, but their effects seemed to be similar to other BPs. The six BPA analogues assessed here presented a similar effect on progesterone secretion compared to BPA in terms of both effect and intensity, even though BPF might exhibit a less potent effect on progesterone secretion compared to other BPs.

3. The cumulative effect of bisphenols on progesterone secretion and on cell proliferation

For the first time, the effect of an equimolar cocktail of seven BPs was assessed on the functional effects of granulosa cells *in vitro*. In the cocktail “ \sum BP 10 μ M”, each BP was at 1.43 μ M, and in the cocktail “ \sum BP 50 μ M”, each BP was at 7.14 μ M. Therefore, the concentration of the cocktail was comparable to individual BPs. The cocktail showed a similar effect on progesterone secretion in terms of intensity, compared to individual BPs or to the mean of effects of the seven BPs, despite each BP being 7-fold less concentrated in the cocktail compared to the seven BPs alone. The seven BPs at 10 μ M reduced progesterone secretion by 17.8–41.3% depending on the BPs, compared to an even greater 47.8% reduction in progesterone secretion after the cocktail “ \sum BP 50 μ M” (7 \times 7.14 μ M) treatment. We confirmed that the decrease in progesterone secretion and proliferation is not due to a cytotoxic effect of the cocktail (“ \sum BP 10 μ M” or “ \sum BP 50 μ M”). As data were normalised with the protein level of the corresponding well, the effect observed in all conditions including the cocktail does not correspond to impairment of the proliferation. These data, therefore, suggested a cumulative effect of the BPs on progesterone secretion. Further study should investigate the potential additivity of these BPs effects. This finding raises the issue of individual regulation of these molecules instead of considering the whole family of molecules.

A significant decrease in hGC proliferation was observed at the 50 μ M concentration of BPAF, BPAP, BPB and the cocktail “ \sum BP 50 μ M”. Previous studies have shown a controversial effect of BPs on cell proliferation. BPA increased cell proliferation in KGN cells (Shi et al., 2021), while it reduced granulosa cell proliferation in ovine (Teteau et al., 2020) and did not affect swine GC (Berni et al., 2019; Grasselli et al., 2010). BPS did not alter hGC proliferation (Amar et al., 2020) but reduced cell proliferation in ewe GC (Teteau et al., 2020) and swine GC (Berni et al., 2019). It seems that the effect of BP depends on the molecule assessed and the model used. Even though no effect of any BP at 10 μ M was observed, an inhibitory effect was reported for the equimolar cocktail “ \sum BP 50 μ M” (7 \times 7.14 μ M). This effect showed a cumulative effect of the seven BPs to impair progesterone secretion and cell proliferation. These results, therefore, highlighted the need to further investigate the potential additive or even synergistic effect between several BPs.

4. Viability and oestradiol secretion

In the present study, viability was assessed by two complementary

methods, and significant differences were highlighted by the CCK8 assay only after 48 h of 50 μM BPAF and BPAP treatment. Such a decrease in cell viability was previously reported in porcine GC after a 10 mM BPAF treatment (Bujnakova Mlynarcikova and Scsukova, 2021a), in the COV434 human granulosa cell line at 100 μM (Bujnakova Mlynarcikova and Scsukova, 2021b) and in KGN cells at 10 μM (Huang et al., 2020). The effect of BPAP on GC was reported for the first time in the present paper and therefore cannot be compared with the literature. Regarding viability assays of the other BPs, these results were relevant to previous data showing that BPA and BPS affected neither proliferation nor viability of GCs at the tested concentration in hGCs (Amar et al., 2020) or in the human KGN granulosa cell line (Shi et al., 2021). In *in vitro* animal models, similar results of an absence of effect of BPA and BPS on cell viability were reported in ovine (Teteau et al., 2020), bovine (Campen et al., 2018), porcine (Berni et al., 2019) and rat (Samardzija et al., 2018) GCs. The difference between the LDH assay (measured in the supernatant) and CCK8 assay (measured in cells) could suggest that the alteration in viability had just started before the 48-h endpoint, explaining why it could be evidenced in cells but not yet in the supernatant, meaning the CCK8 assay is potentially more sensitive than the LDH assay. Another explanation could be that both bisphenols might affect mitochondrial function as BPAF in the KGN cells (Huang et al., 2020) and that this reduction in mitochondrial activity could influence the ability of the cells to reduce the tetrazolium salt in the CCK8 assay, that could explain the decrease in viability observed in the present study. The CCK8 assay was performed after 24 h of treatment to investigate the toxicity of BPAF and BPAP and no effect was reported, confirming that the effect started around 48 h of treatment.

In this study, despite the absence of an effect on oestradiol secretion at 10 μM for each BP, various effects were reported at 50 μM . Indeed, while BPAF and BPAP significantly decreased oestradiol secretion, BPB significantly increased it. No other significant differences were found for the other BPs. Controversial effects on oestradiol secretion have already been reported in the literature. Indeed, BPA and BPS showed a decrease in oestradiol secretion in hGCs (Amar et al., 2020; Mansur et al., 2016) and human KGN granulosa cell lines (Shi et al., 2021). On the contrary, BPA increased oestradiol secretion in mouse GC (Shi et al., 2017) and ewe GC (Teteau et al., 2020). However, these controversial effects could depend on the concentration. In porcine GC, at low concentrations (0.1 μM) BPA stimulated the secretion of oestradiol, while at higher concentrations (1, 10 μM) BPA decreased it (Grasselli et al., 2010; Mlynarcikova et al., 2005; Wu et al., 2018). A decrease in oestradiol secretion was also shown in rat GC (Pogrmic-Majkic et al., 2019; Zhou et al., 2008). The oestradiol plasma level was also shown to be inversely correlated with plasma BPA levels in women undergoing ART (Bloom et al., 2011), which is relevant to an inhibitory effect of BPA on oestradiol secretion. Regarding BPS, it increased oestradiol secretion in sheep GC (Teteau et al., 2020) and bovine GC (Campen et al., 2018), while it decreased it in porcine GC (Berni et al., 2019; Bujnakova Mlynarcikova and Scsukova, 2021a) and in an ovine model of basal folliculogenesis (Vignault et al., 2022). Moreover, the effect of oestradiol seemed to vary in ewes according to the metabolic status of the ewe (Teteau et al., 2022). A decrease in oestradiol secretion was also reported after BPAF treatment in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a). Effects of BPB were not reported in granulosa cells, but a study on male rats also showed an increase in plasma oestradiol levels following exposure to 50 $\mu\text{g/L}$ BPB (Ullah et al., 2019). The effects of BPs on oestradiol secretion could, therefore, vary depending on the BP considered, the concentration, the metabolic status and the species considered. Regarding our results, the inhibitory effect observed after 48 h of BPAF or BPAP exposure could be partly explained by their potential cytotoxic effect. No effect on oestradiol secretion or cell viability was observed after 24 h of BPAF or BPAP treatment. Therefore, the inhibitory effect on oestradiol secretion observed at 48 h cannot be dissociated in our conditions from a toxicity issue.

5. Regulation of gene expression

A decrease in *CYP17A1* and *CYP19A1* mRNA expression after BPAF and BPAP treatment at 50 μM was reported. Such a decrease in *CYP17A1* and *CYP19A1* is relevant to the inhibitory effect of BPAF and BPAP on oestradiol secretion. *CYP17A1* is an enzyme involved in androgen production and *CYP19A1* (aromatase) is an enzyme involved in the transformation of androgen into oestrogen. *CYP17A1* is physiologically expressed in theca cells but not in GC. *In vitro* GC could differentiate a bit from *in vivo* cells and, therefore, express *CYP17A1*. This result could therefore indicate a potential effect on *CYP17A1* in theca cells that could accumulate with an effect on *CYP19A1* in GC and therefore worsened the impairment of oestradiol secretion. BPAF *in vivo* exposition showed a decrease in testosterone level in rat and mice, which is in line with the reduction in *CYP17A1* reported in the present paper (Li et al., 2022; Yu et al., 2022). Nevertheless, the literature reports controversial results, as an exposure to BPAF during both gestational and postnatal period increased testosterone level in rat offspring (Li et al., 2016). Further studies would be required to decipher the mechanism of action in both thecal and granulosa cells. In our previous work, Amar et al. (2020) showed no effect of BPS on *CYP17A1* expression in hGCs, neither at 10 μM nor at 50 μM , which is consistent with the present results. Regarding *CYP19A1*, other studies showed no effect on *CYP19A1* mRNA expression after BPA and BPS treatment in ovine GC (Teteau et al., 2020). At lower BPA concentrations than ours, a decrease in *CYP19A1* mRNA expression was reported in hGCs (Mansur et al., 2016). A decrease in *CYP19A1* mRNA was also found in porcine GC after BPAF and BPS 10 μM treatment (Bujnakova Mlynarcikova and Scsukova, 2021a). The decrease in oestradiol secretion after BPAP and BPAF treatment could be explained by this decrease in *CYP19A1* expression. Nevertheless, a cytotoxic effect of BPAF and BPAP could explain both a decrease in oestradiol secretion and a decrease in *CYP17A1* and *CYP19A1* expression. Nevertheless, because all other genes do not vary after BPAF and BPAP treatment, the effect on gene expression seemed to be independent of a cytotoxic effect.

An increase in *HSD3B2* mRNA expression was reported after treatment with BPB and the cocktail " Σ BP 50 μM " treatments. Such an increase in *HSD3B2* mRNA expression could be a compensation for the inhibitory effect on progesterone secretion. However, it was not reported with all BPs, which is in line with the absence of effect reported on *HSD3B* expression in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a). Contrary to our results, a decrease in *HSD3B* expression was reported after BPA treatment in hGCs and ovine GC (Mansur et al., 2016; Teteau et al., 2020). In the present study, no effect was found on *CYP11A1* mRNA expression with any BP. BPA did not alter *CYP11A1* expression after BPA treatment in porcine GC (Bujnakova Mlynarcikova and Scsukova, 2021a), while in ovine GC or hGCs, a decrease was reported after BPA treatment (Mansur et al., 2016; Teteau et al., 2020). *HSD3B2* and *CYP11A1* are involved in progesterone production, which is impaired by all BPs. However, their expression is not altered by most of BPs in the present study. The fact that *HSD3B2* expression increase in the cocktail " Σ BP 50 μM " could be due to a potential additivity or synergy between BPs but further studies are required to conclude on this possibility.

No other gene had its expression regulated in this study. A previous study reported a decrease in *AR*, *ESR1* and *ESR2* mRNA expression after 100 μM BPA treatment (Teteau et al., 2020). We, therefore, expected to see changes for some BPs, even though only lower concentrations were used in the present study. However, mRNA expression does not necessarily correlate with protein expression. Indeed, a previous study reported a decrease in *CYP11A1* and *HSD3B* mRNA expression without an effect on their protein expression level (Teteau et al., 2020). Regarding the expression of the five candidate genes that were highlighted in a previous RNAseq study characterising gene changes after 1 h BPA and BPS treatment in ovine GC (*JUNH*, *KLF9*, *KLF10*, *LIF*, *TXNIP*) (Teteau et al., 2023), none of them were regulated in hGCs in the present study. This might be explained because differences were not maintained after

24 h of treatment for all these genes, and the species difference might also play a role. Further study should focus on analysing expressional changes in kinetic to decipher BP mechanisms of action.

6. Limits of the *in vitro* study

The limits of the *in vitro* study are partly due to the low quantity of human cells that could be recovered for each culture compared to the number of BPs assessed. Indeed, only two concentrations were assessed in this study, even though it would have been interesting to also assess lower concentrations. Moreover, the chosen concentrations are supra-environmental concentrations. They were chosen for three reasons, first because these doses were already studied on human granulosa cells for BPS (Amar et al., 2020), second, the duration of the treatment is short, 48 h compared to the three months that folliculogenesis lasts, and third because we aimed at investigating whether cumulative mechanisms of action between these molecules could potentially occur, and we, therefore, did not choose to assess environmental levels of BPs. However, previous studies in different cell models and species have shown that GCs are not the most sensitive cells. In fact, an effect of BPS is observed from 10 nM in ovine oocyte and from 1 μ M on the expression of steroidogenesis genes in ovine cumulus cells (Desmarchais et al., 2020), while no effect on ovine GC steroidogenesis was observed below a concentration of 10 μ M (Teteau et al., 2020). The present paper therefore studied the effects of the various BPs at supraenvironmental doses in order to test whether there is a potential cumulative effect on ovarian cells, considering that GCs are not the most sensitive ovarian cells. The results of this present paper therefore suggested that a cumulative effect of bisphenols should be studied on oocyte quality, even though it could be difficult to perform such study using human samples. In addition, repeating such experiments on GCs in a cell culture model allowing a longer culture period and therefore allowing the assessment of lower concentrations would be of interest.

Even though the levels of BPs measured in the women follicular fluid were a lot lower compared to our experimental concentrations, it is important to emphasise that the follicular fluids were recovered after 16 h of fasting. Therefore, the level of BP detected in the follicular fluid is likely underestimated, due to the short half-life of BPs. However, given our experimental results, because BP levels measured in the follicular fluid never exceeded 1 μ M, it is plausible that exposure to bisphenols at environmental levels does not influence steroid production in GCs.

5. Conclusions

In this study, we reported that the seven different BPs had a similar effect on progesterone secretion. For the first time, this study suggested a cumulative effect of BPs on hGC progesterone secretion and on cellular proliferation. Our results highlighted the need to further investigate the potential additive or even synergistic effect between several BPs. Moreover, BPA, BPS and BPF were detected in 23.1% of follicular fluids recovered from women undergoing ART procedures, with some women exhibiting two BPs simultaneously. These findings suggested that a combination of BPs may affect the success of their ART attempt.

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Authors' contributions

MEL performed the experiments, analysed the data and wrote the paper. LW, MG, CB, MB, PP, PJ-G, AD, OT and CV performed the experiments. ML and SU analysed the data. VM, FG and AB helped write the paper. SE conceived the study, performed the experiments, analysed

the data and wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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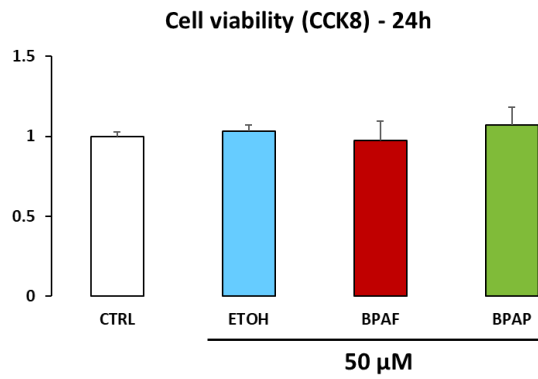
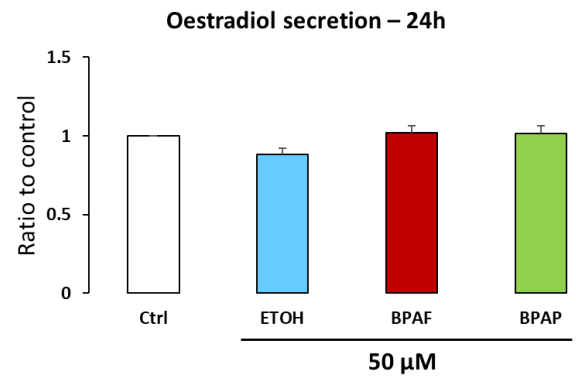
Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.121818>.

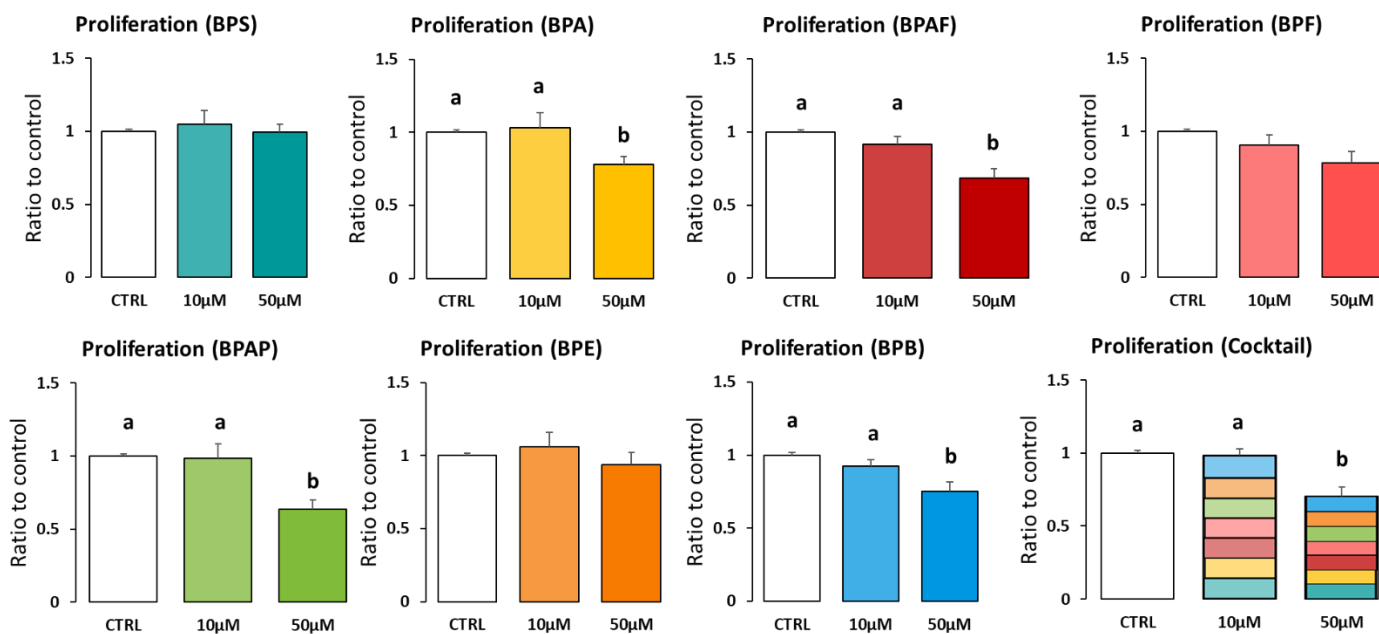
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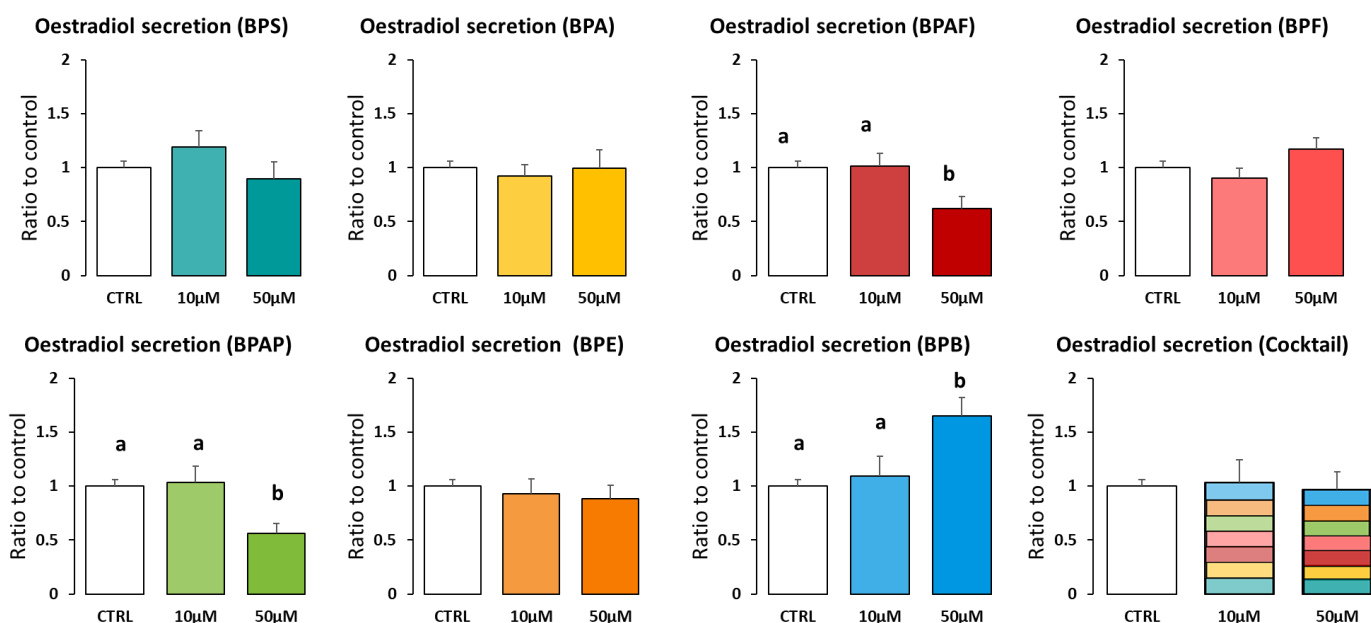
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A**B**

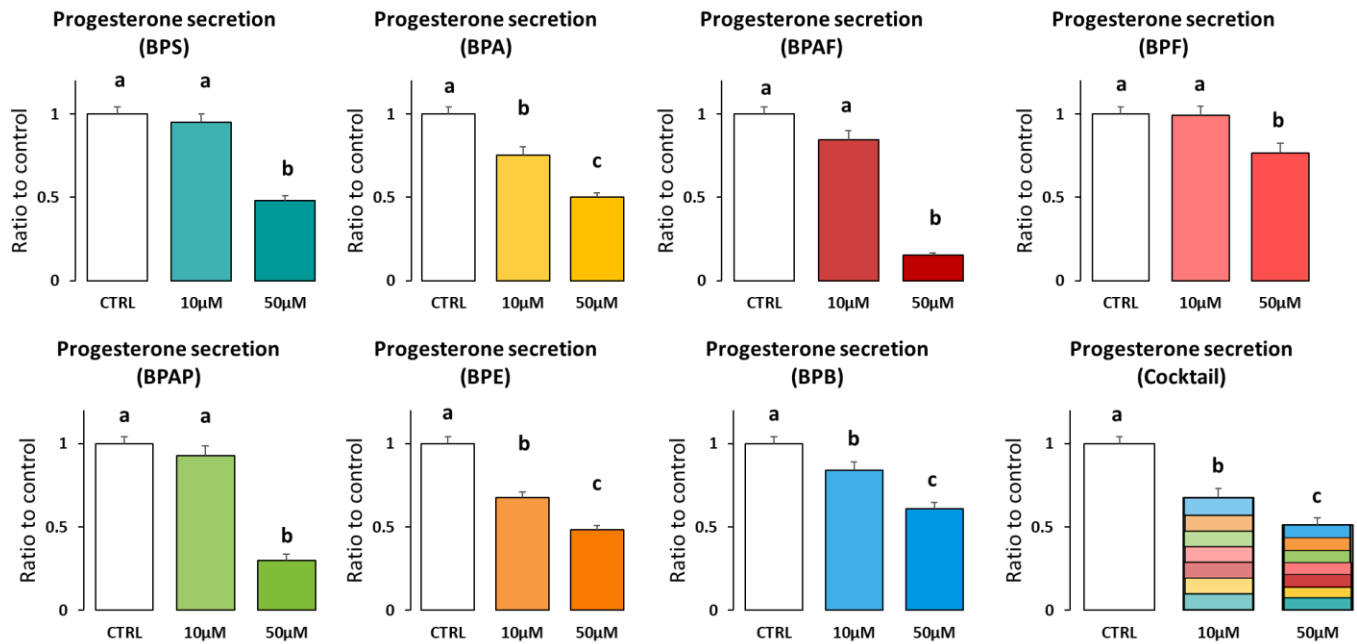
Supplementary Figure 1: (A) Effects of 2 BPs on cell viability after 24 h treatment on human GC in presence or absence of BPAF and BPAP (50 μ M), with CCK8 assay. Significant differences are symbolised by different letters (ANOVA, $p \leq 0.05$). The number of live cells is proportional to the measured optic density. Results are presented as a ratio to the control of each experiment as mean \pm SEM for 7 batches with at least duplicates. **(B)** Effects of BPAF and BPAP on oestradiol secretion were assessed in hGC at 50 μ M. The oestradiol concentration was determinate after 24 h of treatment in culture medium of hGC by Enzyme-Linked Immuno Assay (ELISA). The results were normalised to the control condition of each lot. Data are expressed as pg oestradiol per μ g protein and as mean \pm SEM (12 independents experiment with 2 replicates per conditions). Bars with different letters are significantly different ($p \leq 0.05$).



Supplementary Figure 2: Effect of two concentrations (10 or 50 μM) of 7 different bisphenols on human GC proliferation after 48 h of treatment. HGC underwent 48 h culture with the supplementation of 10 μM bromodeoxyuridine/5-bromo-2'-deoxyuridine (BrdU). The cell proliferation was normalised to the control with ethanol at 1.74 mM condition. The results are expressed as the mean \pm SEM of five independent experiments with four replicates per condition. Bars with different letters are significantly different ($p \leq 0.05$).



Supplementary Figure 3: Effect of two concentrations (10 or 50 µM) of 7 different bisphenols on oestradiol secretion was assessed in human GC after 48 h of treatment. The oestradiol concentration was determined after 48 h of treatment in culture medium of hGC by Enzyme-Linked Immuno Assay (ELISA). The results were normalised to the control condition of each experiment. Data are expressed as pg oestradiol per µg protein and as mean ± SEM (13 independent experiments with 2 replicates per conditions). Bars with different letters are significantly different ($p \leq 0.05$).



Supplementary Figure 4: Effect of two concentrations (10 or 50 µM) of 7 different bisphenols on progesterone secretion was assessed in human GC after 48 h of treatment. The progesterone concentration was measured in culture media of hGC by Enzyme-Linked Immuno Assay (ELISA). The results were normalised to the control condition of each experiment. Data are expressed as ng progesterone per µg protein and as mean ± SEM (15 independent experiments with 3 replicates per conditions, except for cocktail = 8 experiments with 3 replicates per condition). Bars with different letters are significantly different (p ≤ 0,05).

Supplementary Table 1: Relative proliferation and oestradiol and progesterone secretion level in hGC after 48 h treatment with 10 and 50 μ M BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or their equimolar cocktail “ Σ BP 10 μ M” and “ Σ BP 50 μ M”. Regarding the equimolar cocktail, the addition of the seven bisphenols at 7 x 1.73 and 7 x 7.14 μ M reach 10 and 50 μ M, respectively. The results are expressed as mean \pm SEM of five independent experiments with at least duplicates for cells proliferation, 13 independent experiments with two replicates per condition for oestradiol secretion and 15 independent experiments with 3 replicates per condition, except for the cocktail = 8 experiments with 3 replicates for progesterone secretion.

	Proliferation	E2	P4
CTRL EtOH 0.05% (8.6 mM)	1.02 \pm 0.07	1.11 \pm 0.09	1.16 \pm 0.06
CTRL EtOH 0.01% (1.7 mM)	1 \pm 0.02	1 \pm 0.06	1 \pm 0.04
BPS_10μM	1.05 \pm 0.1	1.19 \pm 0.15	0.95 \pm 0.05
BPS_50μM	1 \pm 0.06	0.9 \pm 0.16	0.48 \pm 0.03 *
BPA_10μM	1.03 \pm 0.1	0.92 \pm 0.11	0.75 \pm 0.05 *
BPA_50μM	0.78 \pm 0.06 *	0.99 \pm 0.17	0.5 \pm 0.03 *
BPAF_10μM	0.92 \pm 0.05	1.01 \pm 0.12	0.85 \pm 0.05
BPAF_50μM	0.68 \pm 0.06 *	0.62 \pm 0.11 *	0.15 \pm 0.01 *
BPF_10μM	0.91 \pm 0.07	0.9 \pm 0.1	0.99 \pm 0.05
BPF_50μM	0.79 \pm 0.08	1.17 \pm 0.11	0.77 \pm 0.06 *
BPAP_10μM	0.98 \pm 0.1	1.03 \pm 0.15	0.93 \pm 0.06
BPAP_50μM	0.63 \pm 0.07 *	0.56 \pm 0.09 *	0.3 \pm 0.04 *
BPE_10μM	1.06 \pm 0.1	0.93 \pm 0.14	0.68 \pm 0.03 *
BPE_50μM	0.94 \pm 0.09	0.88 \pm 0.13	0.48 \pm 0.03 *
BPB_10μM	0.92 \pm 0.04	1.09 \pm 0.18	0.84 \pm 0.05 *
BPB_50μM	0.75 \pm 0.06 *	1.65 \pm 0.17 *	0.61 \pm 0.04 *
Cocktail_10μM	0.98 \pm 0.04	1.03 \pm 0.21	0.68 \pm 0.05 *
Cocktail_50μM	0.71 \pm 0.06 *	0.97 \pm 0.16	0.51 \pm 0.04 *

Supplementary Table 2: Relative expression of candidate genes in hGC after 24 h treatment with 10µM BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or their equimolar cocktail “ΣBP 10 µM”. Regarding the equimolar cocktail, the addition of the seven bisphenols at 7 x 1.73 reach 10 µM. The results are expressed as mean ± SEM of six independent cultures.

<i>Gènes</i>	10µM									
	ETOH	BPS	BPA	BPAF	BPF	BPAP	BPE	BPB	Cocktail	Ctrl
AR	10.81 ± 2.11	6.66 ± 2.08	7.43 ± 2.53	4.77 ± 1.39	5.83 ± 2.08	4.24 ± 1.59	5.56 ± 1.35	4.66 ± 0.99	3.61 ± 1.33	3.86 ± 1
BAX	2.84 ± 0.61	2.37 ± 0.45	1.91 ± 0.21	1.69 ± 0.24	2.05 ± 0.4	2.97 ± 1.54	1.76 ± 0.37	2.1 ± 0.63	2.44 ± 0.87	2.74 ± 0.63
BCL2	3.23 ± 1.05	3.89 ± 0.97	3.1 ± 0.74	3.22 ± 0.84	3.01 ± 0.96	3.77 ± 0.87	3.42 ± 1.04	2.87 ± 0.93	4.05 ± 1.66	2.55 ± 0.79
CYP11A1	1.46 ± 0.49	1.37 ± 0.41	0.97 ± 0.2	1.22 ± 0.3	1.3 ± 0.36	1.26 ± 0.25	1.09 ± 0.16	1.45 ± 0.22	1.77 ± 0.48	2.37 ± 1
CYP17A1	4.82 ± 3.5	4.29 ± 2.51	1.4 ± 0.52	3.87 ± 3.01	3.3 ± 2.54	1.21 ± 0.48	0.96 ± 0.25	2.04 ± 0.92	1.94 ± 0.81	2.96 ± 1.03
CYP19A1	11.78 ± 2.96	11.12 ± 5.29	11.78 ± 4.79	8.04 ± 2.37	4.69 ± 1.03	12.55 ± 5.67	10.82 ± 4.1	10.7 ± 4.06	5.82 ± 2.75	5.74 ± 1.43
ESR1	2.44 ± 1.46	0.87 ± 0.14	1.23 ± 0.39	1.51 ± 0.43	0.86 ± 0.09	2.25 ± 1.24	1.12 ± 0.32	1.58 ± 0.49	1.21 ± 0.27	1.63 ± 0.45
ESR2	2.26 ± 0.7	2.44 ± 1.03	1.52 ± 0.41	1.95 ± 0.59	2.02 ± 0.49	1.85 ± 0.52	1.53 ± 0.41	1.72 ± 0.53	1.22 ± 0.37	1.7 ± 0.35
StAR	2.6 ± 1	1.69 ± 0.66	1.69 ± 0.67	1.69 ± 0.68	1.69 ± 0.69	1.69 ± 0.70	1.69 ± 0.71	1.69 ± 0.72	1.69 ± 0.73	1.69 ± 0.74
HSD3B1	3.44 ± 0.89	5.21 ± 1.3	3.99 ± 1.17	3.67 ± 0.71	4.58 ± 0.97	1.44 ± 0.36	2.23 ± 0.54	2.71 ± 0.98	2 ± 0.27	2.58 ± 0.59
HSD3B2	2.95 ± 0.61	2.67 ± 0.51	3.04 ± 0.76	1.92 ± 0.51	3.2 ± 0.92	2.35 ± 0.42	3.02 ± 0.59	2.36 ± 0.4	2.32 ± 0.21	2.14 ± 0.39
JUNH	2.97 ± 0.92	2.56 ± 0.36	2.72 ± 0.36	2.23 ± 0.38	2.88 ± 0.64	1.85 ± 0.55	3.31 ± 1.31	3.11 ± 1.21	3.06 ± 1	2.49 ± 0.47
KLF9	2.5 ± 0.86	1.69 ± 0.53	2.36 ± 0.89	1.45 ± 0.5	1.47 ± 0.46	1.18 ± 0.4	1.73 ± 0.67	1.57 ± 0.46	1.85 ± 0.61	1.96 ± 0.53
KLF10	1.77 ± 0.29	1.66 ± 0.34	2.08 ± 0.38	1.23 ± 0.32	1.93 ± 0.36	1.28 ± 0.39	1.58 ± 0.26	1.32 ± 0.27	2.2 ± 0.41	1.44 ± 0.27
LIF	1.67 ± 0.41	1.05 ± 0.21	1.33 ± 0.22	1.29 ± 0.31	1.04 ± 0.33	0.89 ± 0.3	1.48 ± 0.48	1.54 ± 0.71	1.58 ± 0.51	1.22 ± 0.22
TXNIP	5.3 ± 1.73	5.47 ± 1.73	4.66 ± 0.97	3.57 ± 1.43	4.64 ± 1.44	3.31 ± 1.07	4.26 ± 1.12	4.76 ± 1.71	5.12 ± 2.14	5.07 ± 1.51

Supplementary Table 3: Relative expression of candidate genes in hGC after 24 h treatment with 50µM BPS, BPA, BPAF, BPF, BPAP, BPE, BPB or their equimolar cocktail “ΣBP 50 µM”. Regarding the equimolar cocktail, the addition of the seven bisphenols at 7 x 7.14 µM reach 50 µM. The results are expressed as mean ± SEM of six independent cultures.

<i>Gènes</i>	50µM									
	ETOH	BPS	BPA	BPAF	BPF	BPAP	BPE	BPB	Cocktail	Ctrl
AR	4.37 ± 1.56	4.25 ± 1.7	4.93 ± 1.48	4.92 ± 1.18	6.16 ± 2.26	5 ± 1.55	6.8 ± 3.61	4.25 ± 1.76	6.17 ± 2.3	3.86 ± 1
BAX	3.62 ± 1.68	2.6 ± 0.76	3.24 ± 0.91	5.4 ± 0.74	3.06 ± 0.41	4.03 ± 0.72	4.24 ± 0.68	3.56 ± 0.76	3.21 ± 0.56	2.74 ± 0.63
BCL2	4.39 ± 2.63	4.13 ± 2.07	3.19 ± 1.47	4.23 ± 0.95	3.66 ± 1.11	4.83 ± 1.61	5.05 ± 1.59	3.86 ± 1.19	2.19 ± 0.44	2.55 ± 0.79
CYP11A1	2.98 ± 1.32	4.87 ± 1.68	3.6 ± 1.01	1.45 ± 0.49	4.54 ± 1.6	0.76 ± 0.15	2.13 ± 0.95	2.65 ± 1.1	1.96 ± 0.46	2.37 ± 1
CYP17A1	2.2 ± 0.98	0.89 ± 0.24	3.91 ± 2.73	0.26 ± 0.19 *	0.92 ± 0.34	0.13 ± 0.06 *	0.65 ± 0.23	1.18 ± 0.47	0.6 ± 0.3	2.96 ± 1.03
CYP19A1	10.01 ± 4.2	7.51 ± 3.37	6.53 ± 2.07	1.15 ± 0.74 *	7.13 ± 2.91	1.05 ± 0.56 *	4.39 ± 2.04	6.45 ± 3.35	3.72 ± 1.81	5.74 ± 1.43
ESR1	1.38 ± 0.62	1.34 ± 0.66	2.04 ± 1.15	2.84 ± 1.23	1.65 ± 0.47	1.5 ± 0.77	2.8 ± 1.46	1.7 ± 0.72	1.9 ± 0.68	1.63 ± 0.45
ESR2	1.74 ± 0.42	1.91 ± 0.19	2.64 ± 0.85	1.2 ± 0.27	2.11 ± 0.39	1.39 ± 0.26	1.26 ± 0.13	1.87 ± 0.45	2.2 ± 0.52	1.7 ± 0.35
StAR	2.5 ± 1.43	2.95 ± 2.3	4.08 ± 1.99	6.21 ± 3.14	3.77 ± 2.04	3.26 ± 1.09	4.47 ± 2.39	2.03 ± 0.58	2.49 ± 1.13	2.21 ± 0.96
HSD3B1	2.17 ± 0.52	4.11 ± 1.59	3.83 ± 1	1.93 ± 0.94	4.99 ± 1.1	2.34 ± 0.69	6.33 ± 1.87	5.71 ± 1.94	3.92 ± 0.9	2.58 ± 0.59
HSD3B2	2.34 ± 0.37	3.13 ± 0.34	3.61 ± 0.71	1.6 ± 0.48	4.22 ± 0.94	1.91 ± 0.8	2.97 ± 0.87	4.03 ± 0.95 *	3.87 ± 0.33 *	2.14 ± 0.39
JUNH	2.39 ± 0.61	2.05 ± 0.62	2.45 ± 0.52	4.49 ± 0.83	1.9 ± 0.36	3.11 ± 0.82	2.53 ± 0.45	2.32 ± 0.58	1.86 ± 0.49	2.49 ± 0.47
KLF9	1.34 ± 0.54	1.66 ± 0.91	1.37 ± 0.62	3.29 ± 1.65	1.57 ± 0.63	2.36 ± 0.81	1.44 ± 0.48	1.17 ± 0.38	1.5 ± 0.75	1.96 ± 0.53
KLF10	1.45 ± 0.46	1.23 ± 0.39	1.21 ± 0.27	2.01 ± 0.44	1.42 ± 0.26	1.47 ± 0.39	1.42 ± 0.3	1.46 ± 0.24	1.46 ± 0.25	1.44 ± 0.27
LIF	1.23 ± 0.5	0.82 ± 0.33	1.38 ± 0.69	3.91 ± 1.41	0.78 ± 0.15	1.07 ± 0.19	1.17 ± 0.42	0.91 ± 0.39	0.78 ± 0.21	1.22 ± 0.22
TXNIP	4.86 ± 2.22	3.94 ± 2.11	2.95 ± 1.21	3.11 ± 1.19	3.51 ± 1.44	3.76 ± 0.81	4.07 ± 1.82	4.08 ± 1.6	3.1 ± 1.31	5.07 ± 1.51