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Research Article

Effects of Bisphenols on the Assisted Reproductive Technology Outcomes Considering the Patient Clinical Parameters

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Abstract

Context: Bisphenol A (BPA) and its analogues disrupt endocrine functions, adversely impacting oocyte meiosis, maturation, and granulosa cell (GC) steroidogenesis.

Objective: To identify clinical factors, particularly adiposity and age, influencing ovarian cell sensitivity to bisphenol (BP) exposure.

Methods: This study analyzed a cohort of 368 women undergoing assisted reproductive technology (ART) from 2019 to 2023. Four BPs (BPA, BPS, BPF, and BPAF) were quantified, and ART outcomes (eg, oocyte count, embryo quality, and pregnancy rates) were assessed using regression models. GCs from 156 patients were cultured and exposed to BPS for 48 hours to evaluate progesterone and estradiol secretion based on clinical parameters.

Results: BPS and BPA were the most prevalent BPs in follicular fluid. BP exposure was associated with reduced fertilization rates ($P = .05$). Obesity tended to lower live birth rates ($P = .08$) but did not affect embryo development or implantation. Age significantly impacted embryo quantity ($P < .001$) and quality ($P = .03$). GC progesterone secretion was correlated with donor age after exposure to 1 µM and 10 µM BPS ($P = .03$ for both). GCs from younger women appeared more sensitive to BPS.

Conclusion: Although obesity did not affect embryonic development, its association with reduced live birth rates suggests a suboptimal environment for implantation and/or fetal development. Age was linked to lower antral follicle count, pregnancy rates, and live birth rates. Younger women's GCs may exhibit heightened sensitivity to BPS exposure, warranting further investigation.

Key Words: assisted reproduction, female fertility, endocrine disruptors, bisphenols, ART outcomes, granulosa cells

Abbreviations: AMH, anti-Müllerian hormone; ART, assisted reproductive technology; BMI, body mass index; BP, bisphenol; BPA, bisphenol A; CV, coefficient of variation; FSH, follicle-stimulating hormone; GC, granulosa cell; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; TSH, thyroid-stimulating hormone.

Infertility is estimated to affect between 8% and 12% of reproductive-age couples and is correlated with a significant increase in the number of assisted reproductive technology (ART) procedures performed worldwide [1]. These increases are attributed to several factors, including the age at conception and environmental factors. Indeed, the woman's age at conception has increased over the years. In fact, in 2013, 20% of births in England and Wales corresponded to women aged ≥35 years and 4% to women over 40 years compared with 6% and 1%, respectively, in 1980 [2]. In the European Union, the mean age of first-time mothers has continued to rise from an average of 28.8 years in 2013 to 29.7 years in 2022. It increases every year by an average of 0.1 years [3]. Among the environmental factors that can affect reproductive functions, the prevalence of overweight and obesity more than doubled between 1990 and 2022 according to World Health Organization data [4, 5]. Moreover, the human population is exposed to numerous environmental pollutants, including endocrine disruptors.

Bisphenols (BPs) are a large family of aromatic organic compounds containing 2 phenolic groups, with the main member being bisphenol A (BPA) [6, 7]. The main route of exposure to BPs is through the diet because of their widespread use in food packaging and canned food coatings [8] and of the transfer of molecules present in food packaging to their contents [9, 10]. BPs are also present in consumables used by laboratories dedicated to ART procedures [11]. Several studies have evidenced BPA exposure in various human fluids and tissues, such as follicular fluid, urine, and blood [12-14]. BPA has adverse effects on human health, including impaired thyroid

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and liver function, metabolic diseases such as type 2 diabetes, cardiovascular disorders, hypertension, obesity, and impaired male and female reproductive functions [15-17]. Since its identification as an endocrine disruptor, BPA has been regulated and banned in the food industry in Belgium, Canada, and France [6, 7], and substituted by structural analogues [8]. Several BP analogues have already been detected in human fluids and culture media used in ART [11, 18-20]. Similarly to BPA, several analogues promote obesity and diabetes in humans [21] and impair granulosa cell (GC) steroidogenesis in vitro [20, 22]. In animal models, BPs exhibit adverse effects on the male and female pig reproductive systems and their offspring, notably on steroidogenesis in male testes and female GCs. They also reduce oocyte maturation and promote the production of reactive oxygen species [23]. In vitro, BPs alter steroidogenesis by theca cells in cattle [24], steroidogenesis by GCs in bovine [25], rodent [26, 27], and ovine [28] species; and modulate in vitro oocyte developmental competence in sheep [29]. After a 3-month daily in vivo exposure, bisphenol S (BPS) also altered the steroidome of preovulatory follicular fluid, oviductal fluid, and plasma in sheep [30].

Because BPs impair reproductive function, steroidogenesis, and in vitro oocyte developmental competence in several species, we hypothesized that BP exposure contributes to explain the rise in infertility observed in the human population. Our first aim was to analyze the relationship between BPA, BPS, bisphenol F (BPF), and bisphenol AF (BPAF) levels in follicular fluid and the results of ART procedures in women, while considering the patients' clinical parameters. As BPS is the main substitute for BPA in France, our second aim was to evaluate whether the clinical parameters of patients influence the individual sensitivity of their GCs to the effects of BPS exposure in vitro, in terms of steroidogenesis.

Materials and Methods

Bioethics and Experimental Design

The study involved a prospective cohort of 368 women undergoing ART because they exhibited infertility issues, meaning an absence of clinical pregnancy after 1 year of regular sexual intercourse. The cohort also included oocyte donors and women undergoing fertility preservation. Their GCs and follicular fluid were recovered from oocytes punctured from women undergoing ART at the Reproductive Medicine and Biology Department of the Tours University Hospital. This study was approved by the local review committee of Protection des Personnes, Ile de France II (ID RCB: 2020-A03241-38). Each patient was asked to sign a no prior objection form regarding the use of their GCs. All data were collected and analyzed anonymously for each patient, including administrative data (age, profession) and clinical data (age, body mass index [BMI], hormone assays, in vitro fertilization [IVF] data, ART outcomes, etc.). The clinical data were only collected for 310 women undergoing an IVF protocol who consulted for feminine etiology (41%), masculine etiology (19%), both feminine and masculine etiology (34%), and other (6%, ie, single women, unknown etiology). The remaining 58 women underwent follicular puncture to preserve their fertility or to donate oocytes. We collected their follicular fluid and GCs for the BP assays and in vitro GC treatment.

Collection of Clinical Data

For each patient, the following variables of interest were collected (see Table S1 [31]): administrative data including age (in years), residence, and socio-professional category as defined by the National Institute for Statistics and Economic Studies (INSEE) in 2020. There are 6 socio-professional categories: (1) farmers; (2) craftsmen, shopkeepers and business owners, particularly self-employed women and self-employed or salaried business owners (not including the liberal professions); (3) executives (ie, managers and higher intellectual professions); (4) intermediate professions and employees; (5) manual workers; and (6) unemployed. The following clinical data were collected: height (cm), weight (kg), BMI (kg/m^2), weight changes over the past 1 year and 3 months preceding the ART techniques, and the number of cigarettes smoked per day. The biological data included the results of hormone assays: follicle-stimulating hormone (FSH, IU/L), luteinizing hormone (LH, IU/L), anti-Müllerian hormone (AMH, ng/mL), estrogen (pmol/L), progesterone (nmol/L), prolactin (µg/L), thyroid-stimulating hormone (TSH, mIU/L), and thyroid peroxidase antibodies (IU/mL). It should be noted that these hormones were measured in plasma at varying times of the estrus cycle before the ART procedure; conversely, follicular fluids and cells were collected after hormonal synchronization and ovarian stimulation, and thus all corresponded to the preovulatory stage. In addition, ultrasound data enabling the antral follicles to be counted before the choice of ART technique were evaluated. Data related to in vitro–produced embryos included the number of recovered oocytes at day 0 (–D0, the day of the ovarian puncture and oocyte collection), the number of mature oocytes (D0 for intracytoplasmic sperm injection [ICSI] and D1 for IVF after cumulus cell removal), the number of zygotes with 2 pronuclei (at D1, meaning fertilized oocytes where both sperm and oocyte nuclei are still visible, to confirm fertilization), the number of cleaved embryos, the number of good quality cleaved embryos (according to the regularity of the cells and the absence of cytoplasmic fragmentation), the number of fragmented embryos (at D2), the number of embryos in prolonged culture, the number of useful embryos (either transferred at D2 or D5-6, or frozen at D5-6), and the best quality fresh embryo transfer rate (Fig. S1 [31]). The parameters collected at D2 of culture are expressed as a ratio to the number of mature oocytes (metaphase II; the fertilization and normal fertilization rates, the embryo cleavage rate, the good-quality cleaved embryo rate, the useful embryo rate, and the early pregnancy rate). Data related to IVF outcomes included the early pregnancy rate (defined by a blood test for human chorionic gonadotropin [hCG] ≥ 5 IU/L) at D7 after transfer at D5 or at D10 after transfer at D2), the early pregnancy rate confirmation (defined by hCG level, which must have doubled by the second day after transfer), early pregnancy (hCG level > 1000 UI/L at D9), biochemical pregnancy (in the event of pregnancy, a third blood test is carried out 1 week later and the hCG level should be > 1000 IU/L), clinical pregnancy with a fetal heart beat (diagnosed at 8 weeks of amenorrhea by ultrasound of at least 1 fetus with a discernible heartbeat), and the live birth rate after fertility treatment.

Bisphenol Assays

BPA, BPS, BPF, and BPAF were quantified as glucuronides, a metabolized form, in 368 follicular fluid samples taken from women undergoing ART treatment. Each sample was collected

at the time of oocyte puncture and stored at −20 °C in a glass tube until analysis. The BP concentration measured corresponds to the pool of the 2 to 3 first preovulatory follicular fluid collected so that no blood contaminated the sample. Quantification without a hydrolysis step was performed using 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 the positive electrospray ionization and multiple reaction monitoring mode according to a previously described method [32]. The limit of quantification was set at 0.05 ng/mL.

Chemicals and Antibodies

BPS (CAS number: 80_09_1; purity: ≥99%) was purchased from Merck Sigma-Aldrich (Saint Quentin Fallavier, France). All other chemicals were obtained from Merck Sigma-Aldrich, unless indicated otherwise in the text.

Isolation of GCs and In Vitro Cultures

GCs were retrieved from 368 women undergoing ART, during oocyte punctures at the Reproductive Medicine and Biology Department of the Tours University Hospital, following ovarian stimulation treatment. After centrifugation (5 minutes, 400*g*), follicular fluid was collected and stored at −20 °C until the BP assays. GCs were obtained as described previously (Amar et al [22]). Briefly, the cells were washed and resuspended in ACK (155 mM ammonium chloride, 10 mM potassium bicarbonate and 0.10 mM EDTA) for 3 minutes at room temperature to lyse red blood cells. Then, the GCs were washed again, centrifuged (5 minutes, 400*g*, room temperature) and resuspended in McCoy's 5 A medium supplemented with 3 mM L-glutamine, 0.1% bovine serum albumin, 5 mL of penicillin/streptomycin (10 000 UI/mL), 20 mM HEPES (1 M), 96 nM 4-androstene-11 β-ol-3,17-dione, 5 mg/L bovine apo-transferrin, 0.12 µM selenium, and 1.74 µM insulin. The GCs were purified using a 50% Percoll density gradient centrifugation (30 minutes, 700*g*, room temperature). After another wash in medium and centrifugation (5 minutes, 400*g*, room temperature), the GCs were stained with Trypan blue and counted in the Thoma chamber. Next, 100 000 live GCs per well were plated in 96-well plates at 37 °C overnight prior to treatment. Individual cultures were carried out for 156 patients (156 for the progesterone assay and 117 for the estradiol assay).

GC Treatment

After overnight culture, the GCs were exposed individually to a supra-environmental BPS concentration (1, 10 and 50 µM). BPS was diluted with ethanol, so that there was an ethanol control corresponding to each BPS concentrations (0.001% or 17.1 mM ethanol for 1 µM BPS, 0.01% or 171 mM ethanol for 10 µM BPS, and 0.05% or 856 mM ethanol for 50 µM BPS). The cells were cultured in a humidified atmosphere containing 5% CO_2 in air at 37 °C for 48 hours. After 48 hours, the culture supernatants was collected and stored at −20 °C until progesterone and estradiol measurement.

Progesterone Assay

The progesterone concentration in the culture supernatant was determined using a competitive enzyme immunoassay according to a previously described protocol [33]. The absorbance was measured at 405 nm with a Sunrise basic plate reader (TECAN Life Sciences, Switzerland) and Magellan software (https://lifesciences.tecan.com/software-magellan?p=tab–1, Magellan, Tecan, Switzerland). For a progesterone concentration of 0.25 to 32 ng/mL, the mean intra-assay coefficient of variation (CV) was <7% (mean CV: 1.5%, min: 0.10%, max: 6.1%). Progesterone secreted in each well was normalized by the protein concentration of the same well. The results, expressed as nanograms of progesterone per microgram of protein, were then normalized to the control condition of each experiment, and are presented as the mean ± standard error of the mean (SEM) of 156 experiments with 3 replicates per condition.

Estradiol Assay

The estradiol concentration was determined using an enzyme immunoassay (DIAsource, E2-EASIA-kit, Louvain-La-Neuve, Belgium), according to the manufacturer's instructions. Briefly, 50 µL of culture supernatant was used for the assay; competition between unlabeled and labeled estradiol (present in the culture supernatant and supplied by the kit, respectively) lasted for 2 hours at 4 °C. For an estradiol concentration of 1.56 to 50 pg/mL, the mean interassay CV was 17%. The results, reported as picograms of estradiol per microgram of protein, were normalized to the control in each experiment and are presented as the mean ± SEM of 117 experiments with at least 2 replicates per condition.

Statistical Analysis

Quantitative variables are presented as the mean ± SD, qualitative variables are described as counts and percentages, and all coefficients are described with their 95% CI. All these data are categorized into 3 main groups: patient characteristics (age, height, weight, BMI, etc.), ART outcomes (number of antral follicles, recovered oocytes number, embryo cleavage rate, etc.), and endocrine data (FSH, LH, AMH, estradiol and progesterone levels, etc., see Table S1 [31]). Multiple linear and logistic regression were employed to assess the relationship between ART outcomes and endocrine data as response variable, and BP exposure, age, BMI, and AMH as explanatory variables in the 368 women. Other parameters such as infertility ethology, duration of infertility, previous ART treatment, and gonadal hormone dosage were tested to ensure no bias was induced and that there was no need to add these factors in the statistical model (see Fig. S2 [31]). For continuous outcomes, mean and SD are presented with the linear regression coefficient (β). For qualitative variables, counts and percentages are presented with the odds ratio. All coefficients are described with their 95% CI (β). To reduce any possible bias, the models were adjusted with 4 predictive variables defined beforehand: age, BMI by class (normal: 18-25 kg/m^2; overweight: 25-30 kg/m^2; obese: >30 kg/m^2), AMH by class (low: ≤1 ng/mL; normal: 1-2.5 ng/mL; high: >2.5 ng/mL) and exposure to BPs (detected, undetected). Residuals were checked visually to validate the normality hypothesis. Regarding the in vitro treatment of GCs with BPS, the "corrplot" package (Wei T, Simko V (2024). R package "corrplot": Visualization of a Correlation Matrix. (Version 0.95), https://github.com/taiyun/corrplot) was used to calculate Pearson correlations coefficients between the progesterone and estradiol concentration and the clinical variables. The linearity of the relationship between the variables was checked by plotting a scatter plot

and a linear regression line; the choice of the correlation method used (Pearson or Spearman) was based on the distribution of values. The significance threshold was set at 5%. Statistical analyses were performed with the RStudio software (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/, version 2023.12.1 + 402).

Results

Cohort Description

The cohort comprised 368 women undergoing ART at the service of Medicine and Biology of Reproduction of the CHRU of Tours (France) over 5 years (2019-2023). The characteristics of the women are presented in Table 1. The mean age was 33.3 ± 0.3 years (min-max: 17-43), and the mean BMI was 24.6 ± 0.3 kg/m^2 (min-max: 18-40.6), with a mean weight of 66.4 ± 0.8 kg (min-max: 44-123) and a mean height of 164.0 ± 0.4 cm. Among these women, 19% were smokers (56 women); they smoked an average of 6.2 ± 0.5 cigarettes per day. The socio-professional breakdown was as follows: 9.2% were unemployed; 5.3% were craftswomen, shopkeepers or business owners; 25.4% were managers; 30.6% belonged to intermediate professions (ie, intermediate positions between managers and operatives, clerks or workers); 25.7% were employees; and 3.9% were manual workers. None of them were farmers. Their ovarian reserve, which is indicated by the number of antral follicles and measured prior the IVF protocol, was 22.7 ± 0.8, ranging from 3 to 90. For the 291 patients who underwent IVF or ICSI, the fertilization and normal fertilization rates were 64.4% and 58.9%, respectively, with a corresponding birth rate of 32.2%.

Table 1. Women characteristics

		mean ± SEM
Number of patients		368
Age		33.3 ± 0.3
BMI		24.6 ± 0.3
Weight (kg)		66.4 ± 0.8
Height (cm)		164 ± 0.4
Smokers (%)		19.0%
Daily cigarettes		6.2 ± 0.5
Socio-professional category	Unemployed	9.2%
	Craftsmen, shopkeepers and company managers	5.3%
	Managers	25.4%
	Intermediate professions	30.6%
	Employees	25.7%
	Manual workers	3.9%
	Farmer	0.0%
Fertilization rate (%)		64.4%
Normal fertilization rate (%)		58.9%
Life birth rate after fertility treatment per patient (%)		32.2%
Total BP exposition rate (%)		18.8%
Concentration (nM)		0.47 ± 0.1
Double BP exposition rate (%)		2.5%
Concentration (nM)		0.387 ± 0.1
BPS exposition rate (%)		11.1%
Concentration (nM)		0.602 ± 0.2
BPA exposition rate (%)		9.0%
Concentration (nM)		0.184 ± 0.1
BPF exposition rate (%)		1.1%
Concentration (nM)		0.347 ± 0.2
BPAF exposition rate (%)		0.0%

Abbreviations: BMI, body mass index; BP, bisphenol.

We assessed glucuronidated BPs (BPAg for BPA, BPSg for BPS, BPFg for BPF, and BPAFg for BPAF) in follicular fluid. We detected at least 1 BP in 69 of the 368 samples assessed, yielding a 18.8% detection rate (BPSg in 41 [11.1%] samples, BPAg in 33 [9%] samples, BPFg in 4 [1.1%] samples; BPAFg was not detected in any of the samples). The mean concentration considering all glucuronidated BPs was 0.47 ± 0.11 nM, with a mean 0.60 ± 0.17 nM for BPSg, 0.18 ± 0.05 for BPAg and 0.35 ± 0.15 for BPFg.

BMI Effects on ART Parameters

Table S2 [31] shows the results of ART parameters according to the BMI classes, namely normal (18-25 kg/m^2), overweight (<25-30 kg/m^2), and obesity (>30 kg/m^2). The reference group (named "normal") included women with a BMI of 18-25 kg/m^2. We monitored weight trends for patients for 1 year preceding follicular puncture.

Regarding ART outcomes, all terms are defined in the Table S1 [31]. The mean antral follicle count increased significantly with BMI in the women with obesity ($P = .03$), with a trend for significance in the women who were overweight ($P = .08$). In the women with obesity, the number of recovered oocytes tended to increase ($P = .06$), but there were no significant differences for other oocyte characteristics according to BMI. The mean number of zygotes with 2 pronuclei per patient (indicating good oocyte fertilization) was significantly higher in women with obesity than in the control group ($P = .04$). None of the criteria of oocyte quality (good quality, fragmentation), the early pregnancy rate or confirmation of an early pregnancy, or biochemical and clinical pregnancy with a fetal heartbeat were affected by BMI. Nevertheless, there was a trend towards a lower live birth rate after fertility treatment per patient in women with obesity (17.9%) compared with the control group (34.5%, $P = .08$). Furthermore, there was a significant decrease in circulating FSH and progesterone ($P = .05$ and $P = .02$, respectively) prior to oocyte puncture in women with obesity compared with the control group.

Age Effects on ART Parameters

We ran the regression models with a continuous age variable, but for ease of understanding and interpretation, we dichotomized age at the threshold of 35 years, because advanced maternal age is defined as childbearing in women over 35 years [2]. The results according to age are shown in Table S3 [31]. Several oocyte parameters were significantly decreased in the oldest population, including a decrease in the mean number of antral follicles prior to ovarian stimulation ($P < .001$), the mean number of oocytes recovered ($P < .001$), the mean numbers of mature oocytes ($P < .001$), the mean number of cleaved embryos ($P = .05$), the mean number of good quality cleaved embryos ($P = .03$), and the number of useful embryos ($P = .05$). Nevertheless, the corresponding rates (the mature oocyte, cleaved embryo, good-quality embryo, or useful embryo rates, respectively) were unaffected.

The mean number of zygotes with 2 pronuclei and embryos in prolonged culture decreased in the older women than in the younger women ($P = .04$ and $P = .03$, respectively), while the mean number of fresh embryos transferred increased in the older women ($P < .001$). Although age did not influence the early pregnancy rate, it did significantly decrease the rates of biochemical ($P = .03$) and clinical pregnancies with a fetal heartbeat ($P = .04$), as well as the live birth rate in the older age women (29% in the >35-year-old group vs 34% in the ≤35-year-old group, $P = .02$).

The Effects of BP Exposure on ART Parameters

We ran the regression models with the binary BP exposure variable (detected or undetected); the results according to BP exposure are shown in Table S4 [31]. There were only a few significant effects of BP exposure. Detected individuals exhibited a significant decrease in the fertilization rate (58.4% vs 65.9%, $P = .05$) and a trend towards a decrease in the normal fertilization rate ($P = .07$) compared with the undetected individuals, while the rate of women with successful fertilization rate did not differ between the detected and undetected individuals. There were no other significant effects for any of the parameters.

In Vitro Steroid Hormone Secretion After Exposing GCs to BPS

Before analyzing the data, the absence of correlation between progesterone or estradiol in vitro secretion with the BP level in women follicular fluid was checked. After exposing GCs to BPS (1, 10 or 50 µM) for 48 hours, we measured their progesterone and estradiol secretion and then normalized the levels to the control to define fold changes (Fig. 1). There was a significant decrease in progesterone secretion by GCs exposed to 10 and 50 µM BPS compared with the McCoy control (−17%, [$P < .001$] and −52%, [$P < .001$], respectively) and with the corresponding ethanol controls (−16% for 10 µM BPS compared with 0.01% ethanol [$P < .001$], and −52% for 50 µM BPS compared with 0.05% ethanol [$P < .001$]). There was a significant decrease in estradiol secretion by GCs exposed to 1 µM BPS (−21%, $P < .001$), 10 µM BPS (−21%, $P < .001$), and 50 µM BPS (−36%, $P < .001$) compared with the McCoy control. Only GCs exposed to 50 µM BPS also showed a significant reduction in estradiol secretion compared with its ethanol control (−18%, $P < .001$). Of note, estradiol secretion decreased significantly in GCs exposed to 0.001% ethanol (−12%, $P < .001$), 0.01% (−14%, $P < .001$), and 0.05% (−21%, $P < .001$) compared with the McCoy control.

The Relationship Between Clinical Parameters and Steroid Secretion by GCs After BPS Exposure In Vitro

We compared variations in steroid secretion by GCs after BPS exposure with clinical data from the corresponding patients to investigate a potential relationship between clinical data and the sensitivity of GCs to BPS. We considered the clinical criteria that could influence the response of GCs to BPS: age; BMI; weight; weight change over 3 months prior to IVF or ICSI; BP concentrations if the women were found to be exposed; the number of cigarettes smoked per day; the circulating AMH, FSH, estradiol, and LH levels; the number of antral follicles; the number of oocytes punctured; and the mature oocyte rate. Each criterion was used to determine whether there was a correlation with the fold change in progesterone or estradiol secretion compared with the control at the 3 tested BPS concentrations. We identified 2 variables that appear to modify progesterone secretion by GCs (Fig. 2). Age showed

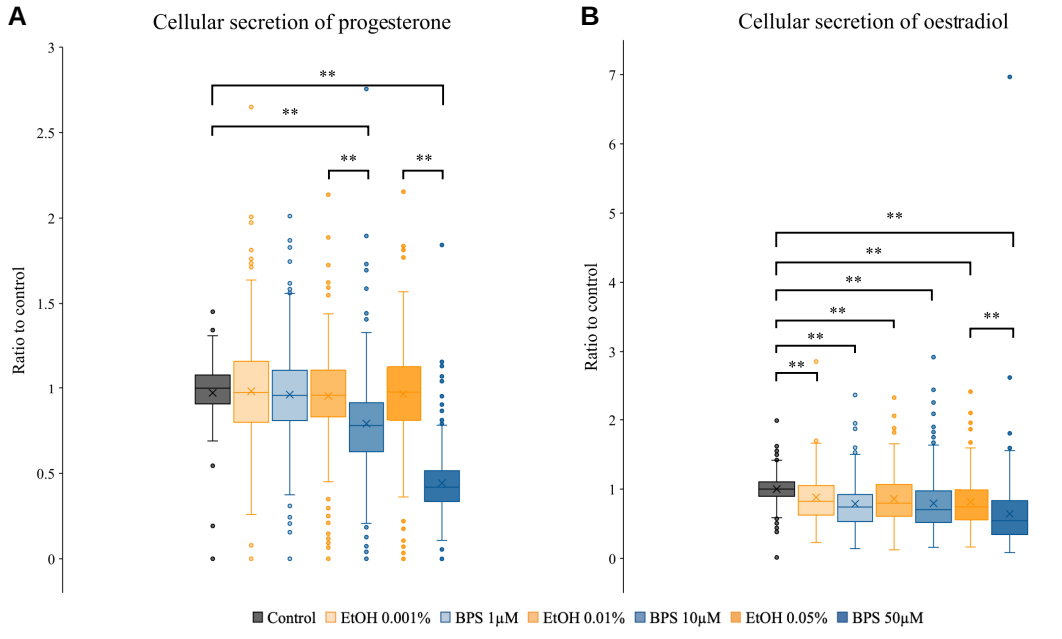

Figure 1. Secretion of progesterone (A) and estradiol (B) by human granulosa cells (GCs) after exposure to bisphenol S (BPS) for 48 hours. Human GC cells were cultured for 48 hours in the presence or absence of 1, 10, or 50 µM. Ethanol controls were also performed to reproduce the potential effect of the ethanol concentration present for each BPS concentration (0.001% or 17.1 mM ethanol for 1 µM BPS, 0.01% or 171 mM ethanol for 10 µM BPS and 0.05%, or 856 mM ethanol for 50 µM BPS). The results are presented relative to the control for each experiment for 156 (progesterone) and 117 estradiol) independent experiments with at least 2 replicates. Asterisks indicate a significant difference, $P < .001$ (analysis of variance, $P \leq .05$). The mean is represented by a cross and the median is indicated by a line inside the boxplot.

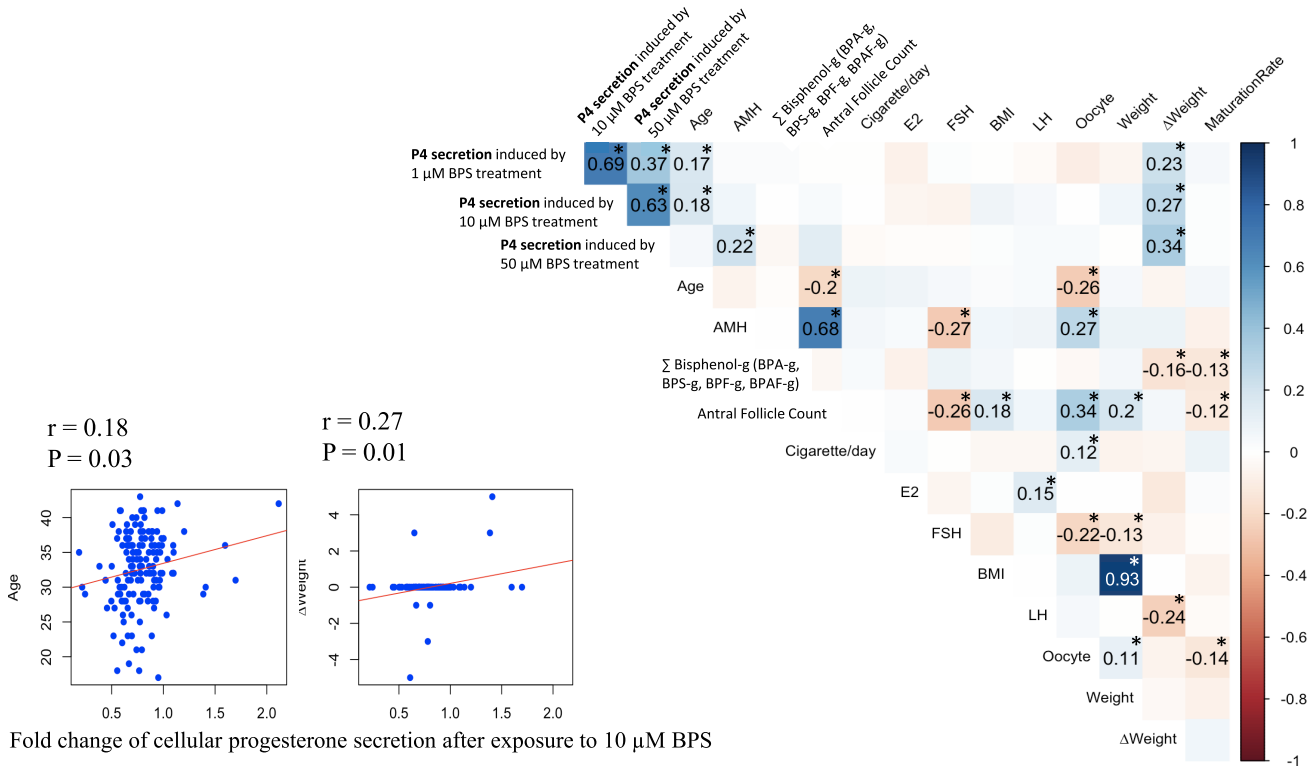

Figure 2. Correlogram of the fold change (FC) in progesterone (P4) secretion by human granulosa cells. Analysis of the correlation between the FC of P4 secretion by GCs after exposure to bisphenol S (BPS, 1, 10, or 50 µM) and clinical data (n = 156). A significant correlation ($P < .05$) is denoted in the corresponding square (Spearman test).

a small but significant correlation with progesterone after exposure to 1 and 10 µM BPS (r = 0.17 [$P = .03$] and r = 0.18 [$P = .03$], respectively). Weight changes over the 3 months preceding the ART procedure also showed a small but significant correlation with progesterone secretion by GCs after BPS exposure to 1 µM BPS (r = 0.23, $P = .03$), 10 µM BPS (r = 0.27, $P = .01$), and 50 µM BPS (r = 0.34, $P = .001$). In addition, circulating AMH levels correlated significantly with progesterone secretion by GCs exposed to 50 µM BPS (r = 0.22, $P = .01$). However, there were no correlations between estradiol secretion by GCs and the clinical parameters (Fig. 3).

Discussion

We investigated the relationships between the levels of BPs in follicular fluid and ART outcomes, according to the clinical parameters of each patient. We found that BMI and age had a greater influence on ART outcomes than exposure to BPs. Advanced age and obesity had an unfavorable effect on the success rate of live birth after fertility treatment. The in vitro experiments involving BPS exposure of GCs enabled us to assess the individual sensitivity to BPS and to analyze the potential influence of each patient's clinical parameters. The significant correlations with age and weight changes suggest that these factors could modulate the sensitivity of GCs to BPS exposure in terms of steroidogenesis.

The Impact of BMI on IVF Parameters

It is well known that the reproductive capacity cannot be optimized without adequate nutrition [34]. Indeed, correlation analyses have shown higher risks of subfertility linked to overweight and obesity in both men and women, and particularly when both members of a couple were overweight [35]. In the present study, BMI and especially obesity were related to a higher number of antral follicles prior to ovarian stimulation and a higher number of recovered oocytes. A meta-analysis of the literature revealed no increase in the follicle count in patients with obesity [36]. Researchers have reported a linear association between a higher BMI (>30 kg/m^2) and a lower number of recovered oocytes [37]. In our study, there was a trend towards an increase in the number of recovered oocytes. Although the number of follicles was higher, their quality was not necessarily improved. Furthermore, studies have shown that obesity, even in metabolically healthy individuals, is associated with unfavorable reproductive conditions and generally with anovulation, infertility, reduced oocyte quality and maturity, as well as with negative impact on conception and implantation [38, 39]. On the contrary, we showed that while the number of recovered oocytes increased in women with obesity compared with women with a "normal" BMI, neither the embryo rates nor their numbers were affected. These results suggest that BMI and obesity have no effects on fertilization and early embryo development steps. Nevertheless, the main result was that the live birth rate tended to be reduced by nearly 2-fold in women with obesity compared with women with normal BMI. This is consistent with the literature. Indeed, a Dutch study of 8457 women reported that women with overweight (BMI > 27 kg/m^2) showed a 33% reduction in the live birth rate after their first IVF treatment [40]. A Chinese study involving more than half a million pregnancies showed that a BMI > 28 kg/m^2 (the threshold for obesity in

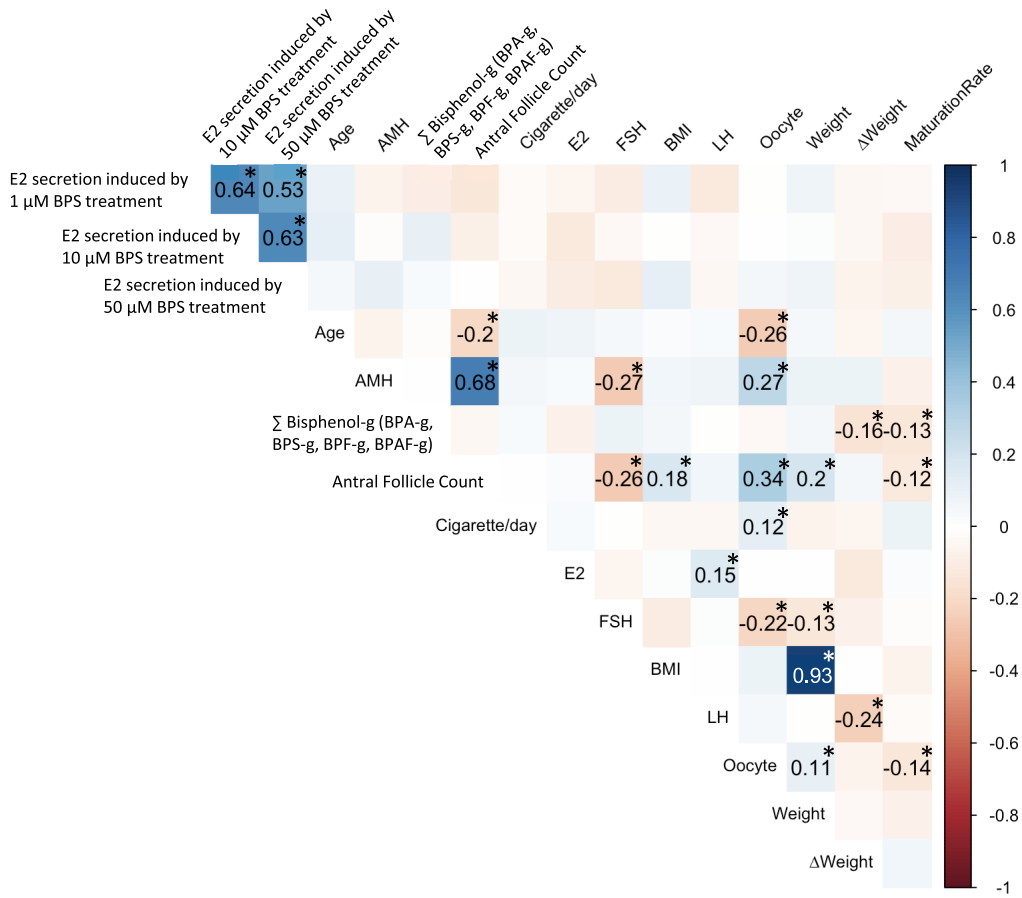

Figure 3. Correlogram of the fold change (FC) in estradiol (E2) secretion by human granulosa cells (GCs). Analysis of the correlation between the FC of E2 secretion by GCs after exposure to bisphenol S (BPS, 1, 10, or 50 µM) with clinical data (n = 117). A significant correlation ($P < .05$) is denoted in the corresponding square (Spearman test).

Asians) was associated with a 16% higher risk of miscarriage [41]. This was further confirmed by another Asian study that reported a higher risk of miscarriage associated with both overweight (8%) and obesity (26%) [42]. Our observation of a reduced live birth rate in women with obesity is in line with the literature reporting a negative impact of obesity on early fetal development [39, 43], a higher risk of first-trimester miscarriage, a lower pregnancy rate and an increased risk of late spontaneous abortion and stillbirth [44, 45]. According to our results, obesity did not appear to impact the clinical parameters of embryos, but the live birth rate tended to decrease, suggesting an environment that is not conducive to fetal development and/or implantation.

The Impact of Age on IVF Parameters

According to our results, advanced maternal age was associated with a lower antral follicle count as well as a lower number of recovered oocytes, findings that are in agreement with the literature [46-49]. These decreases were accompanied by decreases in the number of zygotes with 2 pronuclei, prolonged culture embryos, cleaved embryos, good-quality cleaved embryos, transferred fresh embryos, and useful embryos, but there was no impact on the rate of these parameters. The absence of a correlation between age and the fertilization rate has been reported previously [46, 49]. The significant decreases in the number of zygotes and embryos in our study were likely due to a lower baseline number of oocytes. However, the absence of any effect on the embryo development rates suggests that, in our cohort, advanced age affected mostly embryos in terms of quantity but not in terms of the success of the early steps of embryo development up to the blastocyst stage. Nevertheless, advanced maternal age also impaired embryo quality. Indeed, even though the number of embryos transferred was significantly increased in the advanced maternal age group, the women in this group exhibited a lower biochemical pregnancy rate, a lower clinical pregnancy rate with fetal heartbeat, and a lower live birth rate than the younger women. Our results are consistent with other studies that have investigated embryo morphokinetics in relation to the advanced maternal age effect. Those authors have reported a lower βHCG rate (a parameter used to define successful implantation), a lower pregnancy rate (the presence of a fetal heartbeat), and a lower live birth rate for the advanced maternal age group [46, 49]. Moreover, studies have also shown that embryos from women over 35 years of age with a similar morphokinetic classification are more likely to stop developing and have a significantly lower implantation rate than embryos from women under 35 years of age [49, 50], and their oocytes are similar to those of women with a reduced ovarian reserve [48]. Because age being affects both the quantity and quality of embryos, it is a decisive factor that affects ART outcomes.

BPs Exposure and IVF Parameters

In our study, BP exposure was not related to the ovarian reserve (the antral follicle count), which is consistent with previous studies on BPA exposure [51, 52]. BP exposure was also not correlated with the number of follicle and mature oocytes, and embryo quality, findings that differ from the literature. BPA levels, have been reported to be either inversely correlated with the number of retrieved oocytes [53], or not correlated [54, 55]. In the present study, there was not a significant association between the BP levels in follicular fluid and mature oocytes, which is consistent with a previous study performed on 90 women and considering BPA levels in follicular fluid [56]. On the contrary, a linear dose–response association between increased BPA concentrations in both urine and serum and a decreased number of mature oocytes has been reported in vivo [53, 56] and in vitro when BPA was added during in vitro maturation [57]. Follicular fluid might not be accurate enough to discriminate between exposed and unexposed women, as detailed in the following paragraph, explaining why our results are different from the literature. Therefore, the relationship between BPA levels and mature oocytes could be evidenced in plasma but not in follicular fluid. We found no significant correlation between exposure to BPs and embryo quality, which is consistent with other studies considering BPA levels [53, 58]. In the present study, BP exposure was associated with a reduction in the fertilization rate, which is consistent with previous studies reporting a reduction in the fertilization rate associated with an increase in the BPA urine concentration [17]. However, there was no relationship between the BPA urine concentration and the fertilization rate for 351 women undergoing IVF treatment in a previous study [59]. The in vitro data are consistent with our findings. Indeed, BPS exposure during in vitro maturation of ovine oocytes decreased the in vitro embryo cleavage rate [29].

In our study, we detected at least 1 of the glucuronidated BPs in the follicular fluid of 18.8% of the cohort (69 out of 368 women). The rate of exposed women was probably underestimated as the samples were collected after a 16-hour fasting period whereas the average half-life of BPs is 6 hours. Therefore, at the time of sampling, most BPs had already been metabolized. Furthermore, previous studies have reported that the detection rate for BPs could differ between compartments within the same cohort. For instance, an Italian study assessed BPA levels in 122 patients receiving ART under similar conditions (collection of serum and follicular fluid after overnight fasting); they noted a much lower BPA detection rate in the follicular fluid (28.7%) compared with the serum (52.4%) [60]. Similarly, a Russian study based on 292 patients detected BPA in 16.8% of follicular fluid samples and 92.3% of blood samples from the same patients [61]. Taken together, these data suggest that even though BP measurements in follicular fluid might correspond to women with greater exposure to these compounds, they cannot reliably to discriminate between exposed and unexposed women. Therefore, even though our results on the fertilization rate are consistent with the literature, the conclusions about possible correlations between BP levels and ART outcomes need to be interpreted with caution. To overcome this potential bias, longitudinal exposure measurements should be considered (eg, repeated measurements in urine) to obtain an accurate estimate of the population exposed to BPs [61]. Alternatively, analysis of human hair could allow highly sensitive detection of endocrine disruptors present in the person over the several months preceding the ART procedure [62]. In addition, some IVF media may contain BPs [11], which may complicate the analysis of correlations between BP levels and ART parameters. In fact, the adverse effects observed may not be attributable to the environmental exposure of the patient, but rather to the exposure of the oocytes via their culture medium, or a combination of these 2 factors. Among the BPs measured in the follicular fluid of our patients, the most abundant was BPS (11.1%, 41 patients) with a mean concentration of 0.602 ± 0.17 nM, followed by BPA (9%, 33 patients) with a mean concentration of 0.184 ± 0.05 nM. Although it has been banned in the food industry in France since 2015, BPA is still detected in follicular fluid, which raises questions about the effectiveness of its regulation. Furthermore, a recent study reported sulphated BPA and BPS metabolites in GCs and theca cells. Even though these metabolites do not exhibit any estrogenic activity [63], they could potentially affect cell functioning through other pathways [64]. Additionally, sulphation could contribute to the BP metabolic pathway in the ovary [65]. It would be interesting to measure both sulphated and glucuronidated metabolites to obtain a more accurate exposure assessment.

Correlations Between In Vitro BPS-Induced GC Steroid Secretion and a Patient's Clinical Data

We aimed to determine whether clinical parameters could impact the in vitro response of GCs after BPS exposure. We found that GCs exposed to BPS exhibited a decrease in progesterone secretion, which is in line with previous results in both human [20, 22] and ovine GCs [28, 66]. The decrease in progesterone secretion reported in the present study was related to the patient's age. Indeed, the fold change of progesterone secretion correlated with age, meaning that the lower the fold change of progesterone secretion, the lower the age. Because BPS reduced progesterone secretion, the greatest effect corresponded to the lowest fold change. It might be possible that effect on androgens synthetized by thecal cells could accumulate with effects on steroids produced by GCs, but this was not investigated in the present study. These results suggest that the cells most sensitive to BPS effects secrete less progesterone and belong to younger women. This is a worrying issue because it could mean that younger women are more affected by BPS. The results also showed a positive correlation between weight change in the patients within 3 months prior to IVF and decreased progesterone secretion by their GCs when exposed to BPS in vitro. The women who had lost more weight showed the greatest decrease in progesterone secretion by GCs. Thus, women who have lost weight could be more sensitive to the effects of BPS at the ovarian level. This could be related to changes in lipid and energy metabolism in follicular cells.

BPS can act through several mechanisms [66], including the peroxisome proliferator-activated receptor gamma pathway regulating lipid metabolism [67], which has already been described to regulate steroidogenesis, oocyte maturation, and ovulation [68]. As BPS acts via different mechanisms [66], changes in cellular metabolism could affect the exposure-induced cellular response. Indeed, BPS is able to affect adipogenesis in human adipose-derived stem cells, the 3T3-L1 adipose cell line, HepG2 cells, as well as in mice [67, 69, 70]. Therefore, modulation of lipid metabolism pathways could be very different depending on the cellular environment (eg, weight

loss vs weight gain or absence of weight change). In a weight loss situation, adipogenesis is slowed as the cells are engaged in lipolysis. The promotion of adipogenic pathways in such a lipolytic environment could exert greater effects compared with a cellular environment already engaged in adipogenesis. A metabolism-related mechanism of action could explain why a weight loss situation could contribute to enhance cell sensitivity to the effects of BPS exposure. However, this hypothesis should be further investigated to elucidate the underlying mechanisms involved in the cellular response to BP exposure. Because the promotion of weight loss is one of the first recommendations given to women undergoing ART procedures, the consequences regarding sensitivity to pollutant exposure in the context of weight loss should be studied.

Conclusion

In conclusion, the measurement of BPs in follicular fluid of the patients likely underestimates the exposed population. Hence, it is difficult to correlate ART outcomes with BP detection in follicular fluid from patients without biases. Longitudinal measurements of BPs using either urine or hair and sulfated BP metabolites would provide a more accurate estimation of population exposed to BPs. Nevertheless, we observed correlations between ART outcomes and both BMI and age. Obesity did not impact the embryo development and implantation steps, but the live birth rate tended to decrease, suggesting that the obesity-induced environment is not conducive to proper fetal development and/or implantation. As expected, the age of the patients correlated with a reduction in the antral follicle count, the biochemical and clinical pregnancy rates and the live birth rate. Furthermore, the age and weight changes of women might affect sensitivity of GCs to the steroidogenic effects of BPS, with younger women or women who have lost weight exhibiting more sensitive cells. These results support the need to investigate the relationship between BPS exposure with clinical data and ART outcomes, while considering both age and BMI.

Author Contributions

M.-E.L.R.: Data Curation, Formal analysis, Software, Validation, Writing—original draft, Writing—review and editing; M.B.: Investigation, Writing—review and editing; E.T.: Investigation, Software, Validation, Writing—review and editing; V.M.: Investigation, Writing—review and editing; A.D.: Investigation, Writing—review and editing; S.U.: Investigation, Writing—review and editing; F.G.: Investigation, Writing—review and editing; A.B.: Supervision, Writing—review and editing; S.E.: Conceptualization, Funding Acquisition, Methodology, Project administration, Supervision, Writing—original draft, Writing—review and editing.

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Disclosures

The authors have nothing to disclose

Clinical Trial Information

This study was approved by the local review committee of Protection des Personnes (registered December 15, 2020), Ile de France II and by the Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM, ID RCB: 2020-A03241-38).

Data Availability

The data that support the findings of this study are available from the corresponding author (S.E.), upon reasonable request.

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