A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound

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Bisphenol A (BPA) is a widely studied typical endocrine-disrupting chemical, and one of the major new issues is the safe replacement of this commonly used compound. Bisphenol S (BPS) and bisphenol F (BPF) are already or are planned to be used as BPA alternatives. With the use of a culture system that we developed (fetal testis assay [FeTA]), we previously showed that 10 nmol/L BPA reduces basal testosterone secretion of human fetal testis explants and that the susceptibility to BPA is at least 100-fold lower in rat and mouse fetal testes. Here, we show that addition of LH in the FeTA system considerably enhances BPA minimum effective concentration in mouse and human but not in rat fetal testes. Then, using the FeTA system without LH (the experimental conditions in which mouse and human fetal testes are most sensitive to BPA), we found that, as for BPA, 10 nmol/L BPS or BPF is sufficient to decrease basal testosterone secretion by human fetal testes with often nonmonotonic dose-response curves. In fetal mouse testes, the dose-response curves were mostly monotonic and the minimum effective concentrations were 1,000 nmol/L for BPA and BPF and 100 nmol/L for BPS. Finally, 10,000 nmol/L BPA, BPS, or BPF reduced *Insl3* expression in cultured mouse fetal testes. This is the first report describing BPS and BPF adverse effects on a physiologic function in humans and rodents. (Fertil Steril® 2015;103:11–21. ©2015 by American Society for Reproductive Medicine.)

Key Words: Bisphenol A, bisphenol S, bisphenol F, BPA, BPS, BPF, Insl3, endocrine disruptors, environmental health, fetus, testis, Leydig cells, testosterone, human, organotypic culture, species differences

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BISPHENOL A IS A TYPICAL ENDOCRINE-DISRUPTING CHEMICAL

The field of environmental health emerged historically from the observation of the decreased reproductive success of wildlife populations in relation to industrial chemicals (1). The concept of endocrine-disrupting chemicals (EDCs) was then proposed after various reproductive function alterations in wildlife and humans were linked to the intensive use of pesticides in agriculture (2). Thus, reproduction has a central place in the EDC field. One of the most studied EDCs is bisphenol A (BPA; 2,2-bis

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(4-hydroxyphenol) propane). A survey of the Pubmed database with the use of "bisphenol A" or "BPA" as keywords provided more than 10,000 articles published up to August 2014, including clinical, epidemiologic, and experimental studies. Moreover, BPA played and still has a major role in the emergence of new concepts in the EDC field.

BPA was first synthesized by Dianin in 1891, and its estrogenic activity was discovered in 1936 (3). It is therefore one of the oldest synthetic compounds known for its endocrine activity, although diethylstilbestrol (DES) has stronger estrogenic activity. In the 1950s, it was observed that BPA could be polymerized to make polycarbonate plastic, a miraculous cheap product that is lightweight, transparent, colorable, resistant to impact, heat, and chemicals, inalterable with time, and easy to mold and thermoform. BPA rapidly became one of the most produced and used chemicals worldwide, even though it was a recognized synthetic estrogen. About 70% of BPA production (3.4 million tons per year) is used to produce polycarbonate plastics used in a variety of common products (optical, media, automotive, electrical and electronics, housewares and appliances, construction, medical, packaging, etc.). About 20% of BPA is used as an essential component of epoxy resins that are mainly used to coat the inner surface of food and beverage metallic cans. Finally, BPA is used as antioxidant or inhibitor of polymerization in some plasticizers, polyvinyl chloride, and thermal cash register paper.

BPA can leach from food or beverage containers and is then ingested. This is the main source of contamination, although its ubiquitous distribution leads also to contamination through the skin, especially in the case of thermal paper (4), or via inhalation of household dusts. Its concentration in human serum is a matter of debate, because biomonitoring and kinetic studies have reached seemingly conflicting conclusions (5). Most studies based on the analysis of blood samples from adults found that the concentration of unconjugated BPA, which is the biologically active form, ranges from 0.2 to 10 ng/mL (0.9–44 nmol/L), with an average concentration of ~1–3 ng/mL (4–13 nmol/L) (6–8). However, one study reported that unconjugated BPA represents no more than 2% of the total BPA in blood, leading to a plasma concentration of unconjugated BPA <0.02 ng/mL (<0.1 nmol/L) (9). No data are presently available on the BPA concentration in the plasma of human fetuses during the first trimester of pregnancy. BPA quantification in amniotic fluid and in umbilical cord blood samples during the second and the third trimesters of pregnancy reported a mean level of 1 ng/mL (4.3 nmol/L) with large interindividual variations (10). Furthermore, BPA might particularly accumulate in early fetuses because of lower metabolic clearance or conjugation at that development stage (11).

BPA has been associated with many human diseases, such as diabetes, obesity, cardiovascular, chronic respiratory and kidney diseases, breast cancer, behavioral troubles, tooth developmental defects, and reproductive disorders in both sexes (12–14). Considerable research effort on BPA toxicity during the last decades played a major role in raising two major concepts in the EDC field: low-dose effects (i.e., effects observed for concentrations in the range of human exposure) and nonmonotonicity (i.e., nonlinear relationship between dose and effect, where the slope of the curve changes sign somewhere within the range of the examined doses) (reviewed in 12, 15). Importantly, BPA appears to be one of the EDCs that most frequently shows a nonmonotonic dose-response curve (16).

FETAL TESTIS IS A MAJOR TARGET OF ENDOCRINE DISRUPTORS

One of the most studied functions in the EDC field is male reproduction. Many studies indicate that the incidence of male reproductive function abnormalities in humans has been increasing over the years (17–20). A decline in sperm quality was the first reported alteration (21) and was largely debated. Among the different papers dealing on this issue, published from 1995 up to now and including more than 1,000 participants, 16 found an unambiguous decline in sperm count, whereas ten reported no change and three an increase (22). A recent work showed that sperm concentration in France has been declining by 1.9% per year from 1996 to 2005 (23). It is now generally admitted that, despite geographic variations in semen quality, a global decrease in sperm count has occurred over the past five decades (24). Moreover, the incidence of testicular cancer, which is the most prevalent cancer in young men, has been steadily increasing in all studied countries. For example, it rose from 4 per 100,000 in 1960 to 10 per 100,000 in 2000 in Denmark (25). Finally, although the incidence of cryptorchidism and hypospadias shows large geographic variations (2%–9% and 0.2%–1% of male newborns, respectively), increasing trends have been reported in several countries (26).

In 1993, Sharpe and Skakkebaek hypothesized that EDCs, particularly EDCs with estrogenic effects, could be one of the causes of these disorders (27). This hypothesis has been strengthened by much epidemiologic, clinical, and experimental data over the years (18, 20, 28–31).

According to the "testis dysgenesis syndrome" (TDS) hypothesis, reduced sperm count, testicular cancer, hypospadias, and cryptorchidism result from defaults in testis development during fetal life (32, 33). Specifically, the higher occurrence of hypospadias and cryptorchidism may result from alterations of the function of fetal Leydig cells. Indeed, Leydig cells produce testosterone that is responsible for the masculinization of the male urogenital system and external genitalia (26, 34–37). An important finding in relation to the TDS hypothesis is that androgens must act before the phenotypic masculinization (38, 39). A reduction of testosterone synthesis or action between 15.5 and 18.5 days after conception (dpc) causes masculinization defects, such as hypospadias, cryptorchidism, incomplete development or agenesis of prostate and seminal vesicles, and reduction of the anogenital distance (AGD) and penis length in male rat fetuses, in which morphologic masculinization begins only at 18.5 dpc. It was therefore concluded that, in the rat, the development of genitalia is programmed between 15.5 and 18.5 dpc, a period that was named the "masculinization programming window". If such a window exists in other species, it would be between 13 and 17.5 dpc in the mouse and between the 6.5th and 14th gestational weeks (GW) in

humans. Altogether, the fetal origin of EDC-induced male reproductive disorders and the finding that EDC effects are often more severe when exposure occurs during fetal life than in adulthood (40, 41) suggest the fetal testis as the main EDC target.

BPA AFFECTS FETAL TESTIS DEVELOPMENT

Many studies have shown that, in rodents, exposure to high or low doses of BPA during intrauterine life induces a range of adverse effects in adult testes, including decreased sperm count, DNA damage, and reduced sperm mobility. Although a few experimental works did not observe such effects, the negative effect of fetal exposure to BPA on adult spermatogenesis is highly probable, and a decrease in fertility was observed in the rat (15, 42, 43). Furthermore, perinatal exposure to environmentally relevant doses of BPA induces multigenerational impairments of male fertility (44).

Conversely, the existence of a negative effect of BPA on fetal Leydig cell function or development is more debatable. In the rat, one paper reported a reduction of the AGD (an index of testicular testosterone production) in male pups after gestational gavage with 250 μg/kg/d BPA, but not with lower or higher doses (2.5, 25, and 1,000 μg/kg/d) (45). However, three other independent studies did not find any effect of fetal BPA exposure via maternal gavage with 1–50,000 μg/kg/d (46–48). This was confirmed by a more recent publication showing that 50 or 1,000 μg/kg/d BPA given to pregnant mice did not affect the AGD in the male offspring (49). Moreover, very high doses of BPA, that are not relevant to environmental exposure, were required to reduce rat fetal testosterone production (50). Conversely, in humans, most studies highlighted an association between masculinization defects and BPA exposure during fetal life. In China, sons of workers who were occupationally exposed to BPA during pregnancy showed shorter AGD (51). In the general Korean population, BPA level in the plasma of newborn boys with hypospadias was seven times higher than in newborns without hypospadias, and the difference was statistically significant (52). Finally, although no BPA increase was first detected in newborn boys with undescended testes in France (10), recent data from this group showed a negative correlation between the cord blood concentrations of BPA and insulin-like 3 (INSL3) (53). INSL3 governs the transabdominal descent of fetal testes from their initial mesonephrotic position to the entrance of the inguinal duct (54). We recently partly elucidated these species-specific discrepancies by comparing the direct effect of BPA on fetal Leydig cell function in rat, mouse, and human testis explants. With the use of an organotypic culture system (fetal testis assay [FeTA]) that we developed previously (55–57), we demonstrated that concentrations of BPA as low as 10 nmol/L (2.28 ng/mL) reduce the fetal Leydig cell–specific functions in human fetal testes, but at least 100-fold higher concentrations are required in mouse and rat testes (58).

NEW CHALLENGES ARE RAISED BY BPA

During recent years, BPA regulation has been tightened, particularly to protect against exposure during fetal and neonatal life. For example, BPA was banned in baby bottles in Canada in 2008, in France in 2010, and in the European Union in 2011. The current tolerable dose intake (TDI) is 25 μg/kg/d in Canada and 50 μg/kg/d in Europe. Recently, the French National Agency for Safety in Food, Environment, and Work and the European Food Safety Authority have recommended lowering BPA TDI to 0.1 and 5 μg/kg/d, respectively. Finally, in France, a law will forbid the use of BPA in any food or beverage packaging from January 2015. Therefore, there is an urgent need to find alternatives to BPA for several industrial applications, because BPA is about to be withdrawn from the market.

BPS (2,2-bis [4-hydroxyphenol]sulfone) and BPF (2,2-bis [4-hydroxyphenol]methane) are presently not regulated and are used without restriction. However, BPA-like effects can be hypothesized because their chemical structures are similar (Fig. 1). Toxicological data on BPS and BPF are scarce and no in vivo or ex vivo study has evaluated their effects on any physiologic function in humans or other mammals. One of the aims of the present paper is to bring original data to help address this deficiency.

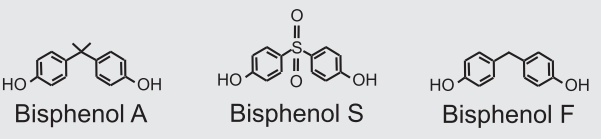
HOW TO ASSESS THE HAZARD OF BISPHENOLS A, S, AND F?

Choice of in Vitro Method

In vitro approaches are simple, fast, and cheap methods to investigate the direct effects of chemicals on fetal testis development and function. They allow reducing the number of killed animals and are an ethically and acceptable way to experiment in humans. They also allow rigorous comparisons between animal models and human samples.

In the 1990s, we started to develop an organotypic culture approach that preserves the testis architecture and intercellular communications. We obtained the best functional and histologic results by placing rat fetal testis explants on a membrane that floats on the culture medium, at the interface between air and medium (55). This procedure allows maintaining in vitro the development of Leydig cells (55), gonocytes (59), and Sertoli cells (60). We then adapted this methodology to the culture of mouse (61, 62) and human (63, 64) fetal testes and named it r/m/hFeTA (rat/mouse/human Fetal Testis Assay). This method was further validated in the present study by showing that the expression of fetal Leydig cell–specific genes (*Star, Hsd3b1, Cyp17a1, Cyp11a1, LH/CG receptor, Insl3*) measured by means of quantitative polymerase chain reaction (qPCR) assay, was maintained in r/mFeTA model (data not shown). FeTA strengths and limits have been previously detailed (56). Briefly, this method allows the precise study of the kinetics

FIGURE 1



Chemical structures of bisphenols A, S, and F.

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and duration of action of specific controlled concentrations of a given compound or mixture of compounds in a defined medium. Treated testis explants are compared with control explants (vehicle alone). Control and treated explants come from the same testis (when fetal testes are large enough to be cut into several pieces, for example in the case of human or late fetal rodent testes) or from the contralateral testis of the same fetus (for 14.5–15.5-dpc rat and 12.5–13.5-dpc mouse fetuses). This paired analysis limits individual variability and increases the method sensitivity. Finally, FeTA is largely less time, money, and labor consuming than in vivo approaches. Like any model, FeTA has inherent limits as well. The main disadvantage is that long-term effects cannot be studied in this system, because testes can develop in vitro only for a few days (4–10 days, depending on the species, developmental stage, and the studied end point). Another drawback is that the FeTA approach allows studying only the direct effect of a compound without taking into account the potential extratesticular feedback loops or actions that could compensate or affect the effects of exogenous chemicals.

Choice of the Age at Explantation

Using this culture system, we often observed clear age-related changes in fetal testis responsiveness to various compounds. For example, GnRH, a putative paracrine testicular factor, does not have any effect on testosterone secretion in 14.5-dpc rat testes, whereas it has negative effects in 16.5–18.5-dpc rat testes and positive effects in testis explants from 20.5 dpc onwards (55). Estradiol and DES reduce testosterone production in 14.5-dpc rat testis explants but not in older specimens (65). Therefore, it is necessary to choose a similar developmental stage when comparing the effect of a compound in different species. We can use human fetal testes from abortions carried out between the 6th and the 12th GW, a period when testosterone secretion and the number of Leydig cells are increasing (66, 67). A comparable developmental stage is 14.5 dpc in the rat (68) and 12.5 dpc in the mouse (62). Interestingly, these stages correspond to the masculinization programming window (38, 39).

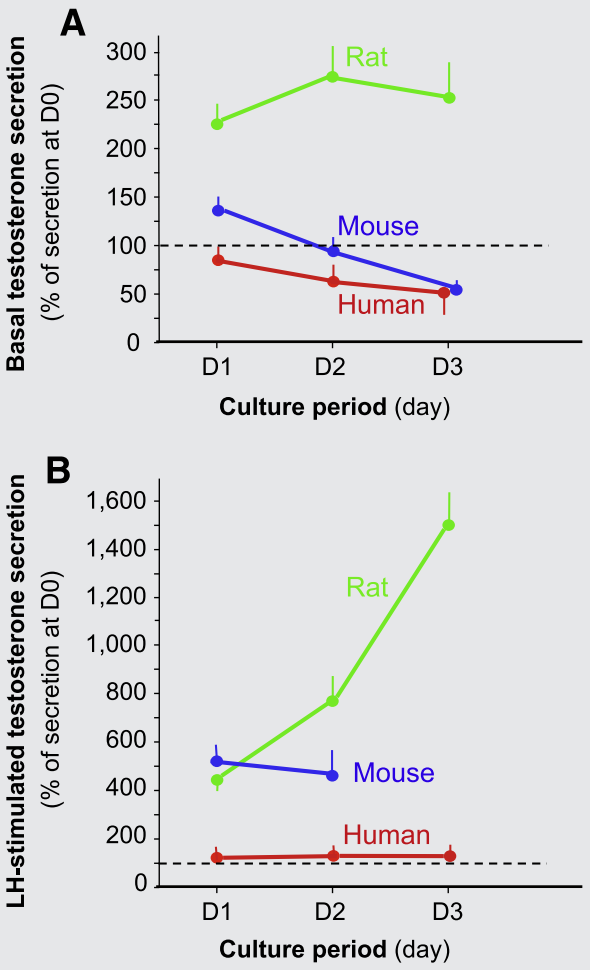
Choice of the Culture Conditions

A key question when evaluating the effect of a compound on fetal Leydig cells using a culture approach is whether this must be done in basal or stimulated conditions (i.e., in the absence or presence of LH or placental chorionic gonadotropin [pCG]). pCG is abundantly secreted during the early stages of human pregnancy; conversely, its expression and activity are not detected in rat and mouse embryos (69–72). In vivo, human fetal testes are stimulated by pCG, so it would seem appropriate to add hCG in the culture medium. However, hCG plasma concentrations change during development and can vary considerably from one fetus to another (73), thus making difficult the reproduction of these in vivo conditions. Stimulation with LH allows the evaluation of the maximal steroidogenic capacity, whereas basal testosterone secretion reflects the testis activity. In vivo and in vitro experiments have shown that the assessment of a compound in basal or stimulated conditions can lead to very different results. For example, ex vivo testosterone secretion by testes from 21.5-day-old rat fetuses that were decapitated in utero 3 or 5 days earlier (a surgical operation that suppresses pituitary LH production and is compatible with embryo survival) was reduced in basal but not in LH-stimulated conditions (74, 75). In vitro, exposure to GnRH increased the basal and reduced the LH-stimulated testosterone secretion by cultured rat fetal testes (55). Similar results were obtained with phthalates in mouse fetal testes (76). Moreover, basal testosterone secretion by rat fetal testes following in utero exposure to diethylhexyl phthalate (DEHP) can be inhibited by DEHP doses much lower than those needed to inhibit hCG-induced testosterone production (77).

Here, we compared basal and LH-stimulated testosterone secretion by rat, mouse, and human fetal testes explanted at comparable developmental stages (14.5 dpc, 12.5 dpc, and 6.3–11.1 GW, respectively) and cultured with the use of the FeTA system for 3–4 days (Fig. 2). Basal secretion rate is presented in Figure 2A. With rat and mouse testes, it increased for 2–3 days and was maintained or decreased thereafter. With human fetal testes, the average of daily basal secretion progressively decreased throughout the culture but individual evolution changed as a function of the developmental stage (63, 64). The ability to respond to LH was higher in rodents than in human, and LH-stimulated testosterone secretion rate increased throughout the culture in the rat testes whereas it was stable in the mouse and human testes (Fig. 2B). These results can be explained by the fact that, as in vivo, each fetal Leydig cell secretes less and less testosterone as a function of the time (62). The species differences in the pattern of evolution of secretion could be explained by the changes in the number of Leydig cells per testis in culture. Indeed, as in vivo, the number of Leydig cells that differentiated during the culture is lower in the mouse than in the rat (62). We did not measure the number of Leydig cells in human fetal testes during the culture. Nevertheless, any increase in their number during the culture time should be very limited, because 3–4 days is a short period compared to the 8 weeks during which human Leydig cells expand in vivo during pregnancy (66, 67). In conclusion, the species differences observed here in the profile of basal and LH-stimulated testosterone secretion in culture are likely to be the consequence of intrinsic species differences in the rate of Leydig cell proliferation and differentiation. Thus, differences between species in the response to one chemical compound observed using the FeTA system can not be attributed to differences in the capacity of this in vitro system to sustain functional Leydig cells.

Using the FeTA system, we previously reported that basal testosterone secretion by human testes was not affected by 10,000 nmol/L DES, but it was reduced by concentrations as low as 10 nmol/L of BPA. Conversely, 10 nmol/L and 100 nmol/L BPA did not affect testosterone secretion by both mouse and rat testes, and 10,000 nmol/L BPA was needed to observe a significant reduction (58). Here, we confirmed and extended these results by showing that 1,000 nmol/L BPA significantly reduced basal testosterone secretion by human and mouse fetal testes (Fig. 3A). Thus, in basal conditions, the lowest observed adverse effect level is 100-fold higher in

FIGURE 2



In vitro testosterone secretion by control rat, mouse, and human fetal testes. Fetal testes were collected from pregnant rat at 14.5 days post-conception (dpc), and mouse at 12.5 dpc as previously described (60). Human testes were isolated from fetuses legally aborted during the first trimester of pregnancy (from 6.3 to 11.1 gestational weeks [GW], mean 8.6 ± SEM 0.3 GW) at the Department of Obstetrics and Gynecology, Antoine Béclère Hospital, Clamart (France) as previously described (63), after obtaining written informed consent for the scientific use of the fetuses. None of the terminations was done for reasons of fetal abnormality. The project was approved by the local Medical Ethics Committee and by the French Biomedicine Agency (reference no. PFS12-002). Fetal testes were cultured with the use of the fetal testis assay system, a method in which the explants are placed on Millicell-CM Biopore membranes (Millipore) floating on 320 μL phenol red–free Dulbecco modified Eagle medium/Ham F12 (1:1) supplemented with 80 mg/mL gentamicin without addition of any exogenous signaling factor (55, 56, 62, 63). Culture medium was completely changed every 24 hours. A whole mouse or rat testis was put on the membrane, whereas human testes were cut into small pieces owing to their larger size and 3–4 pieces/testis were randomly placed on the membrane. Explants were always cultured (**A**) without LH the first day (D0) and then without (basal conditions) or (**B**) with 100 ng/mL LH from human pituitary (≥5,000 IU/mg; Sigma; LH-stimulated conditions) for the 3 subsequent days (D1 to D3). The daily amount of testosterone secreted in the culture medium was measured in duplicate by radioimmunoassay as previously described (68). The limit of detection was 8 pg/100 μL. The intra-assay and interassay coefficients of variation were 2% and 5%, respectively. The daily testosterone secretion at D1 to D3 was divided by the D0 secretion of the same sample to obtain the relative testosterone secretions shown in the figure. Values are shown as the mean ± SEM of 14 rat, 102 mouse, and 27 human fetal testes in basal conditions and 22 rat, 24 mouse, and 10 human fetal testes in LH-stimulated conditions. Values of the amounts of testosterone secreted at D0 were 51 ± 4 (n = 36) and 38 ± 3 (n = 126) pg/testis/h for rat and mouse testes, respectively. For human testes, secretion at D0 changed considerably as a function of time. Values, expressed as pg/testis/h, were 133 ± 17 at 6.5–7.4 GW (n = 7), 1,966 ± 221 at 7.5–8.4 GW (n = 14), 5,808 ± 685 at 8.5–9.4 GW (n = 8), and 12,677 ± 1,950 at 9.5–11.1 GW (n = 8). Because bisphenol A (BPA) can be leached in the medium from the plastic culture material (96), we previously checked that the BPA concentration remained undetectable in control medium at the end of the culture.(58)

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FIGURE 2 Continued

mouse than in human testes and 100- or 1,000-fold higher in rat than in human testes. We also evaluated the effects of BPA on LH-stimulated testosterone secretion (Fig. 3B). In the presence of 100 ng/mL LH, a concentration that induces the maximum steroidogenic response (78), the inhibitory effect of BPA in human testes was much less pronounced than in basal conditions. Indeed, only 10,000 nmol/L BPA (and not all the concentrations equal or up 10 nmol/L BPA as in basal conditions; Fig. 3A) significantly reduced testosterone secretion. This is in accordance with recent results presented at the 18th European Testis Workshop (79). Thus, the maximum capacity to produce testosterone in response to LH is probably not affected by environmental concentrations of BPA in human. Conversely in rat fetal testes, 10 nmol/L BPA had a significant effect in the presence of LH. Similarly, Akingbemi et al. reported that 0.01 nmol/L BPA, but not higher doses, decreased LH-stimulated testosterone production by adult rat Leydig cells in culture, and that this effect was not present in basal conditions (80).

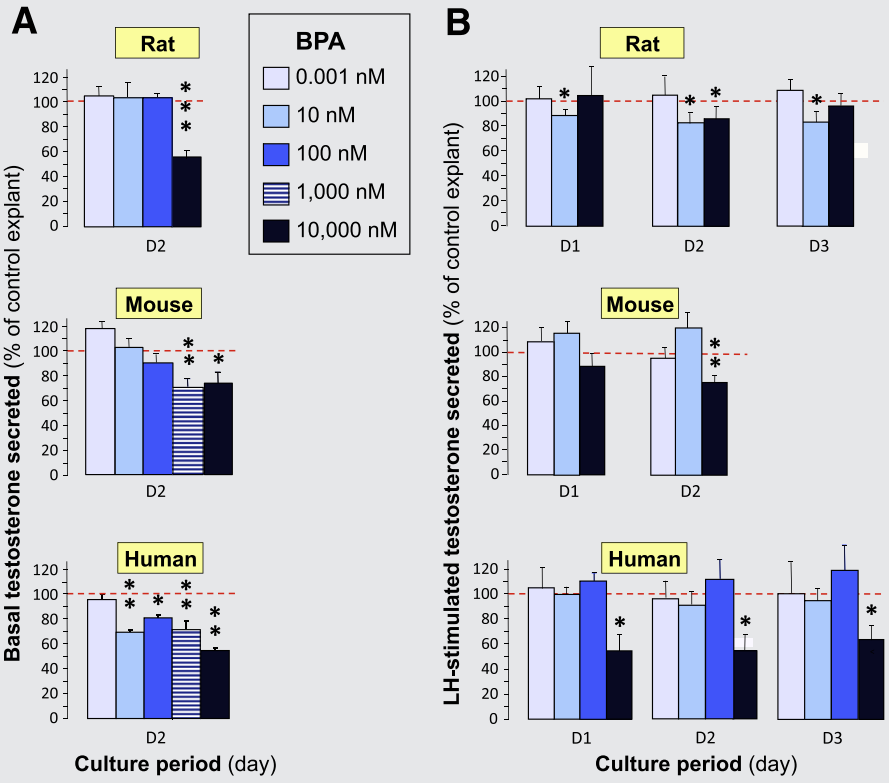
In conclusion, the FeTA method in basal conditions is more sensitive for testes from human and mouse but not from rat, because addition of LH reduced the antiandrogenic effect of BPA in human and mouse testes, whereas it potentiated this effect in the rat. Thus, the use of basal conditions for health risk assessment gives the highest degree of protection for human health. Moreover, the results we obtained in basal, but not in LH-stimulated, conditions are in agreement with bibliographic data suggesting that low doses of BPA affect fetal Leydig cell functions in human, but not rat, testes (see "BPA Affects Fetal Testis Development" section, above). Consequently, we decided to assess the effect of BPS and BPF with the use of the FeTA system in basal conditions with human and mouse fetal testes.

BPS AND BPF ARE NOT SAFE ALTERNATIVES TO BPA

Effect of BPS and BPF on Testosterone Secretion and Comparison with BPA

Using the mouse FeTA model in basal conditions, we tested BPF and BPS effects on testosterone secretion (Fig. 4A). We

FIGURE 3



Effect of bisphenol A (BPA) on (**A**) basal and (**B**) LH-stimulated testosterone secretion by rat, mouse, and human fetal testes. Rat (14.5 days post-conception [dpc]), mouse (12.5 dpc), and human (6.3–11.1 gestational weeks) fetal testes were cultured with the use of the fetal testis assay system as described in the legend to Figure 2. After 24 hours in control medium (D0), explants were cultured in the presence of vehicle alone (ethanol, control explants) or BPA (CAS no. 80-05-7; Sigma; BPA-treated explants) for the three subsequent days (D1 to D3). BPA concentrations ranged from 0.001 nmol/L to 10,000 nmol/L. Control and treated explants came from the contralateral testis of the same fetus (rat and mouse) or from the same testis (human). From D1 to D3, the medium contained (**A**) no LH (basal secretion) or (**B**) 100 ng/mL LH from human pituitary (≥5,000 IU/mg; Sigma; LH-stimulated secretion). Control and BPA-treated explants were from the same mother. The daily amount of testosterone secreted in the medium was measured by means of radioimmunoassay, and the values at D1–D3 were divided by the secretion at D0 from the same sample to obtain relative values. Results are presented as the mean ± SEM of n ratios of the relative secretion of the BPA-treated explants to the relative secretion of the corresponding control explants. Rat: 7–12 fetuses (except for 0.001 nmol/L BPA in LH-stimulated conditions, where n = 4); mouse: 9–17 fetuses; human: 6–8 fetuses (except for 0.001 nmol/L BPA in basal and LH-stimulated conditions, where n = 4 and 5, respectively). *P<.05; **P<.01; ***P<.001 (compared with untreated controls by means of the Wilcoxon nonparametric paired test). Basal values at D2 (**A**) are from N'Tumba-Byn et al. (58) completed with new data for 1,000 nmol/L BPA in human and mouse testis explants.

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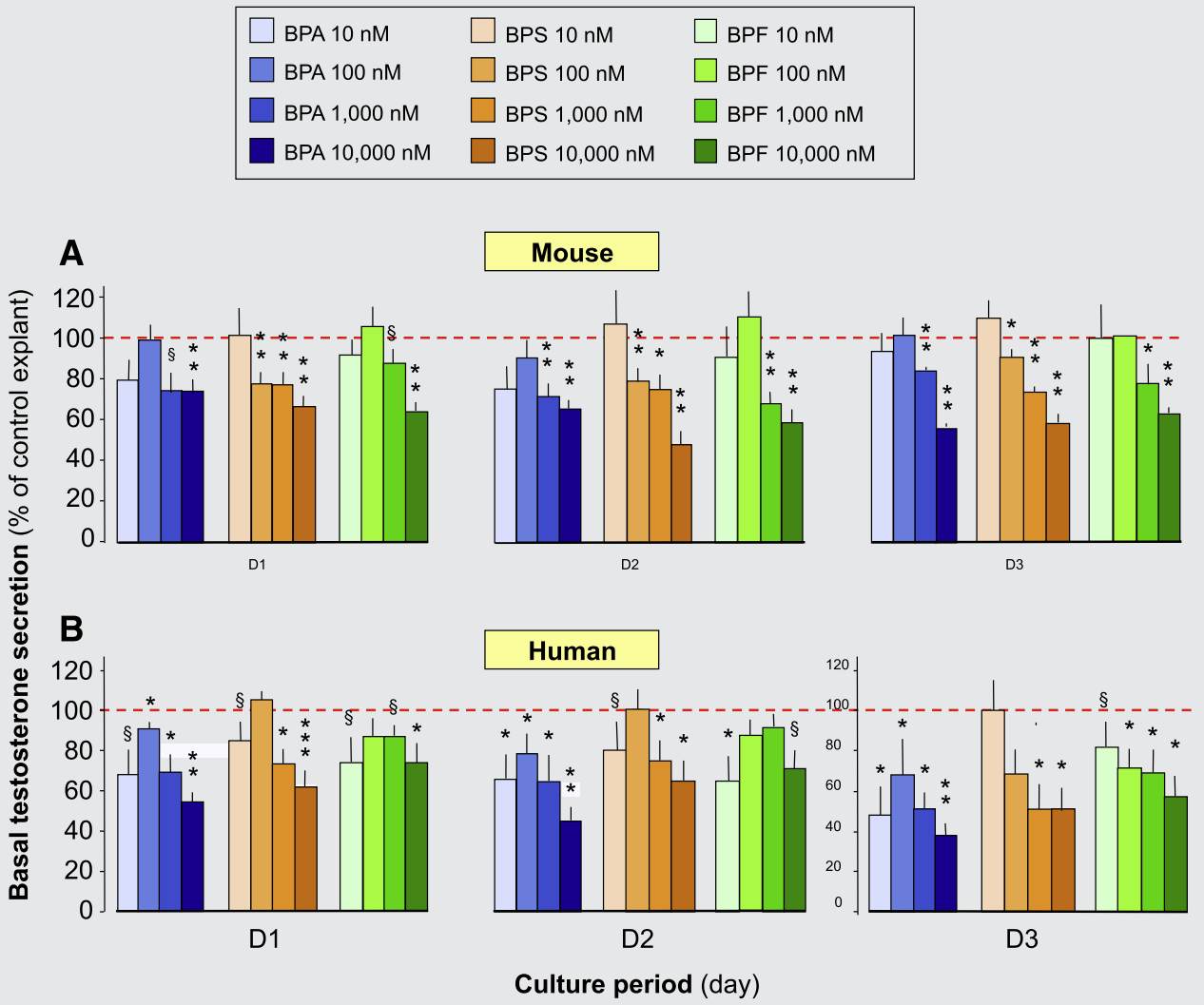
found that BPF showed the same dose-response curve as BPA with a reduction in the amount of testosterone secreted by mouse fetal testes starting from 1,000 nmol/L. BPS had even a more potent inhibitory effect than BPA. Indeed, 100 nmol/L BPS significantly reduced testosterone secretion during the 3 days of treatment, whereas no change was observed with 10 nmol/L and 100 nmol/L BPA.

With the use of the human FeTA model in basal conditions (Fig. 4B), we first confirmed the nonmonotonic profile of the dose-response curve for BPA previously observed in human fetal testes (58). Indeed, the inhibitory effect on testosterone secretion of 10 nmol/L BPA (i.e., the average BPA concentration found in human blood samples in most, but not all studies) was higher than the effect of 100 nmol/L, whereas the effect increased as a function of BPA concentration from 100 to 10,000 nmol/L. Similarly, BPS and BPF reduced the basal testosterone secretion in cultured human fetal testes and showed antiandrogenic effects already at 10 nmol/L with nonmonotonic dose-response curves at days 1 and 2.

This is the first report showing that BPS and BPF may be harmful for one physiologic function in both humans and rodents. Presently, there are no epidemiologic data on the link between BPS or BPF exposure and human health effects. Overall, there are very few data concerning the risk assessment of BPS and BPF. BPS was reported to decrease testosterone production and *Cyp17a* and *17βhsd* mRNA levels in zebrafish (81). However, no experimental study on in vivo mammal exposure or in mammalian organ/primary cell cultures has been published so far. The available data are all issued from cell lines in culture. It was reported that the capacities of BPS and BPF to activate estrogen receptors alpha (ERα) and beta (ERβ) are, respectively, 5- and 10-fold lower

FIGURE 4



Comparative effects of bisphenols A (BPA), S (BPS), and F (BPF) on basal testosterone secretion by (**A**) mouse and (**B**) human fetal testes. Mouse (12.5 days post- conception) or human (6.3–11.1 gestational weeks) fetal testes were cultured with the use of the fetal testis assay system as described in the legend to Figure 2. After 24 hours in control medium (D0), explants were cultured in the presence of vehicle (control explants), BPA, BPS (CAS no. 80-09-1) or BPF (CAS no. 620-92-8; all from Sigma) at concentrations ranging from 10 nmol/L to 10,000 nmol/L for the three subsequent days (D1 to D3). As in Figure 3, control and treated explants were from the same fetus (mouse) or the same testis (human). As in Figure 3, results are presented as mean ± SEM of the relative secretion of treated explants divided by the relative secretion in the corresponding control explants. Mouse: 9–17 fetuses; human: 6–10 fetuses. §$P<.1$; *$P<.05$; **$P<.01$; ***$P<.001$ (compared with untreated explants by means of the Wilcoxon nonparametric paired test).

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than that of BPA in the HELN cell line (82). However, it must be noted that, although this result is important, its scope for environmental health is limited because BPA does not probably physiologically act by directly binding to the classic ERα and ERβ (8). Indeed, its affinity for these receptors is very low (affinity constant Kd of 0.2 and 0.04 μmol/L, respectively) (83). Furthermore, the antiandrogenic effect of BPA is maintained after ERα genetic invalidation, differently from DES, suggesting that BPA effect is mediated by another receptor (58). With the use of the human hepatoma cell line Hep3B, it was reported that BPS could not reproduce various effects of BPA, such as inhibition of the hypoxic response, intracellular lipid increase, pregnane X receptor activation, and changes in the expression of many lipid metabolism genes (84, 85). The E-screen test, an assay to determine the estrogenic activity of chemical compounds with the use of the estrogen-sensitive human breast cancer cell line MCF-7, showed that BPA and BPF estrogenicity were similar, whereas BPS had a weaker effect (82). In a rat pituitary cell line, cell proliferation was activated only by fento-to nanomolar concentrations of BPS and was reduced by these very low BPS concentrations in the presence of E_2 (86). BPF was less cytotoxic than BPA in the HepG2 human hepatoblastoma cells; however, BPF had genotoxic effects that were not observed with BPA (87). BPS

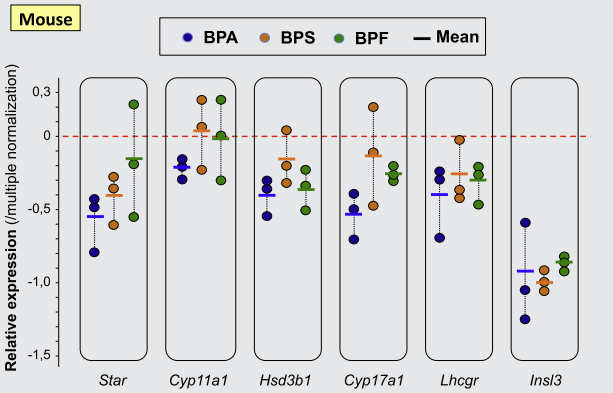
showed similar or more potent effects compared with BPA on lipid metabolism in 3T3-L1 adipocytes (88), whereas BPF effect was lower than that of BPA on adiponectin production and secretion (89). Finally, BPS and BPF reduced the steroidogenic activity of the adrenocortical carcinoma cell line H295 R, but their potencies were, respectively, 5- and 10-fold lower than that of BPA (90). Collectively, these data show that BPS and BPF often display BPA-like activity in cell lines with weaker, equal, or higher potency than BPA, depending on the model and the end points. It is therefore urgent to assess their toxicity with the use of experimental models that are closer to physiologic reality.

The impact of a bisphenol on human health depends not only on its potential hazard, but also on the exposure level. Because BPS and BPF uses are not regulated, it is difficult to list the consumables containing and leaching these compounds. It is known that BPS is often used as a BPA alternative in "BPA-free" thermal printing paper (91). There are few quantitative data on BPS and BPF human exposure. In the USA, the bisphenol analogues predominantly found in foodstuff are BPA and BPF and they account for, respectively, 42% and 17% of all bisphenols (92). In a recent study, BPS was detected in 81% of urine samples collected in the general population in the USA and in various Asian countries (91, 93). Concentrations ranged from 0.02 (limit of detection) to 21 ng/mL (80 nmol/L), and the geometric mean was 0.17 ng/mL (0.7 nmol/L). This varied among countries, and the highest geometric means were observed in Japan and the USA. Thus, the internal concentration of BPS is probably roughly 10-fold lower than that of BPA, but BPS human exposure should largely increase if BPA is gradually replaced by BPS. Furthermore, from the environmental health point of view, one must consider the possibility of additive effects of the various bisphenols.

Effect of BPA, BPS, and BPF on Fetal Leydig Cell–specific Gene Expression

Finally, we compared the effects of 10,000 nmol/L BPA, BPS, and BPF on the expression of fetal Leydig cell–specific genes in 12.5-dpc fetal mouse testis explants (Fig. 5). BPA, BPS, and BPF tended to reduce the expression of all key genes involved in testosterone biosynthesis (*Star, Hsd3b1, Cyp17a1*), except *Cyp11a1*, although statistical significance was never reached owing to experimental variability and the limited number of samples. The three bisphenols also decreased (again not significantly) the expression of *Lhcgr* (the gene encoding the LH/CG receptor). This is not in contradiction to the finding that BPA effect on maximum LH-stimulated testosterone secretion is weaker than that on basal secretion, because the number of LH/CG receptors is not a limiting factor for maximally LH-stimulated testosterone secretion (66, 67). Finally, *Insl3* expression appeared also to be affected by exposure to the three bisphenols, in agreement with *INSL3* down-regulation previously observed in human fetal testes incubated with 10^{-8} mol/L BPA (58). Importantly, we confirmed by means of classic qPCR method that BPS and BPF exposure decrease the expression of *Insl3* by 43 ± 3% and 44 ± 2%, respectively ($P = .062$ [Wilcoxon test]; n = 5; data not shown).

FIGURE 5



Effect of 10,000 nmol/L bisphenol A (BPA), S (BPS), and F (BPF) on the expression of Leydig cell–specific genes in mouse fetal testes. Testes from 12.5-days-after-conception mouse fetuses were cultured with the use of the fetal testis assay system. One testis was cultured in basal medium with vehicle (ethanol, control testis) and the contralateral testis in the presence of 10,000 nmol/L BPA, BPS, or BPF for 3 days (treated testis). The procedure to measure gene expression was previously described (58). Total RNA from a pool of 6–8 testes was extracted with the use of the RNeasy Micro-kit (Qiagen), and 300 μg was reverse-transcribed with the use of the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems–Life Technologies). Then, Taqman low-density array analysis was performed on a 7900HT real-time polymerase chain reaction system, and the expressions of six Leydig cell–specific genes were quantified. Gene expressions were analyzed with the use of the ΔΔCt method and normalized with the use of the geometric mean of two reference genes (*Gapdh* and *18S*). Gene expressions in treated gonads were calculated relative to the specific contralateral control testis. Data are shown as individual values of three pools and are the average log2 of the relative quantification from each experiment. No significant differences were found between treated and control testes with the use of the Wilcoxon nonparametric paired test.

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This is the first comparison of BPA, BPS, and BPF effect on the expression of genes involved in the steroidogenic pathway and Leydig cells differentiation. Earlier data were restricted to BPA and did not permit drawing clear conclusions. BPA gavage led to a reduction in LH/CG receptor protein level in adult-type Leydig cells (94). Conversely, it did not change the expression of *Star, Cyp11a1, Hsd3b1, Cyp17a1,* and *Lhcgr* in mTCL1 cells (mouse Leydig tumor cells) (95), but it reduced the expression of *Cyp17a1* in rat adult Leydig cells in culture (80)and in H295R human adrenocortical carcinoma cells (96). Future studies should determine more precisely whether BPS and BPF activate similar pathways in fetal testes and might thus have additive effects.

CONCLUSION

This paper highlights the need of rigorous comparisons of EDC hazards in human and rodent models in agreement with our previous warning (56, 57).

It shows how BPA is again at the forefront of EDC research because its ban or restriction has raised the question of how to replace it. In this study, we used our

in vitro organotypic culture system to confirm that concentrations of BPA that are environmentally relevant according to most (but not for all) biomonitoring studies, reduce the basal androgenic activity of human fetal testis during a crucial period for masculinization. We also observed that rat and mouse fetal testes are at least 100-fold less sensitive to BPA than human fetal testes in the same conditions. We then demonstrated that in basal conditions (in which fetal testes are most sensitive to BPA effects), BPS and BPF, which are gradually replacing BPA, have antiandrogenic effects that are similar to those of BPA. This is the first report showing a harmful effect of BPS and BPF on a physiologic function in humans and rodents. Because there would be no value in trading one health hazard for another, we should urgently focus on the human health risk assessment of BPA substitutes.

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