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Bisphenol F has different effects on preadipocytes differentiation and weight gain in adult mice as compared with Bisphenol A and S

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Abstract

Bisphenol S (2,2-bisulfone, BPS) and Bisphenol F (2,2-bis [4-hydroxyphenol]methane, BPF) are analogs of Bisphenol A (2,2-bis[4-hydroxyphenyl]propane, BPA), a widely used endocrine disrupting compound present in polycarbonate plastics, thermal receipts and epoxy resins that line food cans. Here we examined effects of BPA, BPS, and BPF in low concentrations on differentiation in murine 3T3-L1 preadipocytes. We also fed adult male mice chow with one of three doses of BPF (0, 0.5, 5, 50 mg/kg chow, or approximately 0.044, 0.44 and 4.4 mg/kg body weight per day) for 12 weeks, collected body weights, food intake, and tested for glucose tolerance. The doses of BPF used produced mean concentrations of 0, 6.2, 43.6, and 561 ng/mL in plasma. In 3T3-L1 cells BPS had the greatest effects, along with BPA, both increased expression of several genes required for preadipocyte differentiation over 12 days in culture. In contrast, BPF decreased expression of several genes late in differentiation. This dichotomy was also reflected in lipid accumulation as BPA and BPS treated cells had elevated lipid concentrations compared to controls or cells treated with BPF. Male mice fed either the highest or lowest concentrations of BPF gained less weight than controls with no effects on glucose levels or glucose tolerance. Plasma levels of BPF reflected doses in food with no overlap between doses. In summary, our results suggest that BPS has a strong potential to be obesogenic while effects of BPF are subtler and potentially in the opposite direction.

Keywords

Bisphenol; metabolism; fat; BPF; alternatives to BPA; diabetes

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1. Introduction

Public concern about potentially harmful health effects of Bisphenol A (2,2-bis[4hydroxyphenyl]propane, BPA) has led to its removal from many plastic products, particularly related to babies and infants (Chapin et al. 2008; Huang et al. 2012; Myers et al. 2009; Vandenberg et al. 2010). In its place, untested, structurally similar compounds are being employed. Two of the most common analogs are Bisphenol S (2,2-bis[4hydroxyphenol]sulfone, BPS) and Bisphenol F (2,2-bis [4-hydroxyphenol]methane, BPF). BPS is used for a variety of industrial applications, and it is present in thermal paper (Siracusa et al. 2018). Currently, BPS is found in up to 81% of human urine samples, collected in the US and Asia. In urine concentrations average 0.654 ng/mL (Liao et al. 2012a). BPF is used to make epoxy resins, vinyl and coatings (Rochester and Bolden 2015; Siracusa et al. 2018) and was recently detected in mustard (Zoller et al. 2016). In a US population, 60% of individuals had detectable BPF in their urine with a range between 0.15–0.54 ng/mL (Ye et al. 2015). As BPA is phased out of use exposure to both these bisphenols will increase (Andra et al. 2015; Eladak et al. 2015; Liao et al. 2012b; Ye et al. 2015).

In rodent studies, neonatal exposure to low levels of BPA increased body fat and weight in offspring (Gao et al. 2016; Junge et al. 2018). Recent reviews of *in vitro*, rodents, and, human data on the relationship between BPA and obesity with or without type II diabetes, also tend to support the hypothesis but point out discrepancies and the need for more data (Stojanoska et al. 2017; Wassenaar et al. 2017). Less is known about the actions of substitutes. Adult male mice exposed from gestation through weaning to BPS had lower body weight as compared with controls (Ivry Del Moral et al. 2016). Adult rats treated with BPF by oral gavage for 28 days had lower body weights than controls (Higashihara et al. 2007). Doses of BPF greater than 20 mg/kg/day also resulted in signs of sickness and toxicity (e.g. decreased locomotion, stained lower abdomen, white turbid and reddish urine) and sex-specific effects on serum cholesterol and glucose. In primary human adipocytes high and low doses of BPA, BPS and BPF changed mRNA and microRNA expression (Verbanck et al. 2017). In murine 3T3-L1 preadipocytes BPS induced lipid accumulation and upregulated key adipogenic markers, as did BPA (Ahmed and Atlas 2016; Helies-Toussaint et al. 2014). In a similar study BPA inhibited adiponectin production and secretion into media, BPF was significantly less effective than BPA (Kidani et al. 2010). Here we compared the effects of these three bisphenols, using low concentrations, on differentiation in murine 3T3-L1 preadipocytes. To follow up on the effects of BPF *in vitro* we conducted an *in vivo* study in which male mice were fed one of three low doses of BPF for 12 weeks.

2. Material and Methods

2.1 3T3-L1 differentiation into adipocytes

3T3-L1 (CL-173) mouse embryonic fibroblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA) repository. As the generation number was unknown, the batch received from the ATCC was designated as passage “0” (P0). For cell propagation, a basal media (BMI; DMEM with high glucose, 10% newborn calf serum, 100 units of penicillin and 100 µg/mL streptomycin) was used before differentiation was initiated. P3 generation cells were seeded on 12-well plates at a density of 6×10^4 cell/cm²

in BMI media, medium was changed at 24 and 72 hours. Cell differentiation was induced six days after seeding the cells by applying differentiation media I (DMI; DMEM with high glucose, 10% fetal bovine serum, 100 units of penicillin and 100 µg/mL streptomycin, 1 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine (IBMX), 0.25 µM dexamethasone). At the same time appropriate wells were exposed to 10 nM, or 1 µM of BPA (CAS#80–05-7), BPF (CAS#80–09-1), or BPS (CAS#620–92-8). Stock solutions of all tested bisphenols (100 mM) were prepared in absolute ethanol and afterwards diluted in PBS. The final concentration of ethanol in the culture was 0.005% for 1 µM and 0.00005% for 10 nM of bisphenol exposure. For a positive control, cells were treated with 2 µM rosiglitazone (ROS). Two days into differentiation DMI was replaced with differentiation media II (DMII; DMEM with high glucose, 10% fetal bovine serum, 100 units of penicillin and 100 µg/mL streptomycin, 1 µg/mL insulin) and 48 hours later media was replaced with basal media II (BMII), which we continued to replace every other day until day 12. Each time the medium was changed, freshly prepared bisphenols were added to evaluate their effect on differentiation (Zebisch et al. 2012).

After 12 days of differentiation, cells were fixed in 4% paraformaldehyde, washed with isopropanol and incubated with Oil Red O (ORO). Following incubation, cells were washed with PBS to remove unbound ORO. For quantitation, ORO bound to stained cells was extracted. Absorbance of the extracts was measured at 490 nm and the amount of ORO was quantified using a calibration curve for ORO in range 23–1,500 µg/mL (Kraus et al. 2016).

2.2 RNA isolation, reverse transcription and quantitative PCR

For qPCR, RNA was isolated from the cells on culture days 0, 2, 4, 6 and 12, treated with DNase I (Invitrogen) to eliminate genomic DNA, and converted into cDNA using SuperScript II (Invitrogen). qPCR (Fast Start SYBRGreen and StepOne Plus system; Applied Biosystems) was performed to evaluate the expression of two transcription factors: peroxisome proliferator-activated receptor gamma (*Pparγ*) and CCAAT/enhancer-binding protein (*Cebpa*), and four adipogenic markers: adipocytes protein 2 (*aP2*, (*Fabp4*)), perilipin (*Plin*), adiponectin (*Adipoq*), and adipisin (*Cfd*). Primers were designed to span exon-exon junction, except for *aP2* and *Plin*, which are single exon genes and their sequences are shown in Table 1. Each set of primers was initially tested for efficiency (between 95–105%) and specificity, using melting curve analysis to verify that one amplicon was represented by a single peak. For data evaluation comparative Ct method was used. Each sample was analyzed in triplicate. Expression levels of target genes were normalized to an endogenous control, 60S ribosomal protein L19 (Rpl19). A calibrator sample was run on each plate to adjust for plate-to-plate variation. Samples with Ct values >35 cycles as well as outliers identified as samples with values above (or below) the 1.5-fold of the interquartile range from the third (or the first) quartile were excluded from the analyses.

2.3 BPF exposure, food intake and body weights adult male mice

Animals and general husbandry—Forty-eight ICR:Crl (CD1) males (age 6–7 weeks at arrival; Charles River, Raleigh, NC, USA) were housed in groups of six animals with food (Envigo Teklad2020, Madison, WI, USA) and water *ad libitum*, room lights were on a L:D cycle of 12:12 (lights on at 1200h). One week after arrival animals were moved to individual

cages and at weekly intervals two mice per group were moved onto their randomly assigned diets. Males received food containing doses of 0, 0.5, 5, or 50 mg of BPF per kg food (0mg-TD.95092, 0.5mg-TD.160864, 5mg-TD.160865, 50mg-TD.160866, Envigo Teklad, Madison, WI, USA) for twelve weeks. Because mice were exposed for such a long period route of exposure via food instead of daily gavage was chosen to minimize stress. Given the lack of impact of treatment on food intake (see results section), there was no concern about inconsistency of exposure. Once the mice started on these diets food intake and body weights were measured weekly, two hours prior to lights out. Males were randomly assigned to groups with approximately equal body weight distribution (12 per group). During weeks 8–12 all mice were subjected to the same regime of behavioral assays: open field, elevated plus maze, and mating tests. Males were mated for 2–7 consecutive days, until we noted a mating plug. During the 4-hour tests water, but not food, was available. There were no differences associated with BPF dose in any of the behavior tests or in the numbers of males that plugged females after 2–7 tests. However, due to the individual variation in numbers of mating test days we decided to include only the first 7 weeks of food intake in this analysis. After mice had been on control or BPF diets for 10 weeks of they were tested for glucose tolerance. All animal care and procedures were approved by the NCSU animal care and use committee and in accordance with AALAC standards.

2.4 Glucose Tolerance Tests

Animals were fasted for 10 hours, starting in the second half of dark phase. Body weights were recorded before and after fasting. At the beginning of the light phase a baseline blood glucose measurement was taken, mice were injected with glucose (ip 2 g/kg body weight). Blood samples were collected 15, 30, 60, and 120 minutes after the injection by tail bleed. Blood glucose was measured using One Touch Ultra2 glucometer (Johnson & Johnson).

2.5 Detection of BPF in plasma

Animals continued on their diets for one week after glucose tolerance tests, then blood was collected to evaluate the amount of BPF in plasma. Samples were collected in the middle of the dark phase to increase the amount of free BPF in blood. Randomly selected males (6 per food condition) were sacrificed by carbon dioxide inhalation followed by decapitation. Trunk blood was collected into heparinized tubes, spun and plasma was collected and frozen. Plasma samples were sent to RTI International and assays of total BPF by LC-MS were conducted blind to treatment groups.

Standard curve spiking solutions were prepared in acetonitrile. The standard curve for LC/MS/MS analysis consisted of concentrations ranging from 2 to 2000 ng/mL. Internal standard ($^{13}\text{C}_{12}$ -BPF) was added at 5 μL per sample from a solution in acetonitrile:water (50:50) at a concentration of 1 $\mu\text{g/mL}$. Twenty-five μL of plasma was aliquoted into a 96-well Waters Acquity 1 mL plate. Five μL of internal standard and 5 μL acetonitrile were added. Twenty-five μL of β -glucuronidase/sulfatase enzyme solution (prepared by mixing 4.01 mg of β -glucuronidase (*Helix pomatia*, Sigma Lot# SLBB1602V) with 96.5 mg of Sulfatase (*Helix pomatia*, Sigma Lot#081M7023V) in 6 mL of 2 M ammonium acetate, at pH 5 was added. Samples were sealed, vortexed (1200 rpm for 3 min), and incubated at 37 °C for 2 hours. Following incubation, the plates were centrifuged (4000 rpm for 6

minutes) and 100 μ L of the supernatant was transferred to a new plate and mixed with 25 μ L of water. For standards, 25 μ L of blank rat plasma was mixed with 5 μ L of spiking solution containing each compound, 5 μ L of internal standard, and 25 μ L of enzyme solution and incubated as per samples. Standard curves were run prior to, in the middle of, and after plasma samples. Quality control samples were included in the middle of the standard curve brackets. Standards were within 15% of nominal or 20% at LOQ (2 ng/mL). Only total BPF was measured.

Analysis was conducted using a Waters Acquity UPLC coupled with an API 5000 Triple Quadrupole Mass Spectrometer with a Turboion Spray source, using a Waters Acquity UPLC HSS-C18, 1.8 μ M, 2.1 $\times$ 50 mm with an Acquity UPLC HSS-C18, 1.8 μ M, 2.1 $\times$ 5 mm Vanguard Pre-column. Elution was conducted with a water(A): acetonitrile(B) gradient at 0.4 mL/min, starting at 20% B for 0.5 min, a linear gradient to 90% B from 0.5 – 2.5 min, held at 90% B for 1.5 min, and returned to 20% B. BPF was monitored by MRM 199 $\rightarrow$ 93, and $^{13}\text{C}12$ -BPF by MRM 211 $\rightarrow$ 99.

2.6 Statistical analysis

Data from differentiated preadipocytes were analyzed using One-way ANOVA for each individual bisphenol on each day. Food intake was analyzed with repeated measure ANOVA with diet as the main factor and week as the repeated measure. Initial body weights were evaluated using One-way ANOVA with diet group assignment as the factor. To account for differences in initial body weight, data collected after diets were started were evaluated using repeated-measures ANCOVA with weekly food intake as a dependent variable, diet as a factor, initial body weight as a covariate, and week as the repeated measure. Glucose tolerance data (area under the curve) were analyzed with One-way ANOVA. Fasting glucose levels and area under curve from glucose tolerance tests were evaluated using One-way ANOVA with diet group assignment as the factor. Post hoc tests using Bonferroni corrections were used when significant main effects or interactions were present.

3. Results

3.1 Expression of adipogenic markers and transcription factors were less affected by BPF than BPA or BPS

In general BPF had less effect on the genes we assayed while BPA and BPS increased their expression, particularly early on (Days 2 and 4) during preadipocyte differentiation. On the first sampling day (Day 2) either one or both concentrations of BPS increased transcription of *Ppar γ* ($F(2,8) = 7.32$), *Cebpa* ($F(2,8) = 7.21$), *Cfd* ($F(2,8) = 6.02$), and *Adipoq* ($F(2,8) = 24.31$) above control levels ($p < 0.025$ at least for these comparisons). Neither BPA nor BPF had any effects this early in the differentiating period. On Day 4 all six genes were enhanced above control levels by one or both concentrations of BPS: *Ppar γ* ($F(2,9) = 5.46$), *Cebpa* ($F(2,9) = 12.47$), *aP2* ($F(2,9) = 7.32$), *Plin* ($F(2,9) = 9.01$), *Cfd* ($F(2,9) = 8.37$), and *Adipoq* ($F(2,9) = 7.40$), $p = 0.03$ at least for all comparisons. In addition, the higher dose of BPA increased gene transcription of all the genes except *Ppar γ* , *Cebpa* ($F(2,9) = 6.89$), *aP2* ($F(2,9) = 8.60$), *Plin* ($F(2,9) = 11.96$), *Cfd* ($F(2,9) = 16.46$), and *Adipoq* ($F(2,9) = 9.49$), $p < 0.02$ at least for all comparisons. The only significant effect of BPF, relative to controls,

was on Day 12 when *Cfd* ($F(2,9) = 4.28$), and *Plin* ($F(2,9) = 4.31$), $p < 0.05$ in each case mRNA were decreased, relative to controls, by the lower BPF concentration (Figure 1).

3.2 Accumulation of lipids in 3T3-L1 cells was enhanced by BPA and BPS but not by BPF

Quantitative analysis of ORO associated with lipids in mature adipocytes showed that both concentrations of BPA and BPS elevated lipid concentrations above controls and cells incubated with BPF ($F(6,21) = 69.80$, $p < 0.001$). The higher concentration of BPS produced intermediate amounts of lipid, higher than controls, but lower than the cells treated with either dose of BPA ($p < 0.05$, Figure 2).

3.3 BPF consumption had no effect on food intake but lowered body weights

Despite random assignment to diets there was an initial body weight difference between the diet groups ($F(3,44) = 2.97$, $p < 0.05$). Male mice in the BPF 50 mg group weighed less at the start of the experiment than controls ($p < 0.05$). To account for the differences in initial body weight, the data were expressed as cumulative body weight gain for each week. We evaluated body weights at three time points: 0, 7 and 12 weeks (Figure 3). A main effect of diet ($F(3,44) = 3.18$, $p < 0.05$) and an interaction between diet and week ($F(3,44) = 3.25$, $p < 0.05$) were present. Mice eating the control diet gained more weight than mice on low (0.5 mg) and high (50 mg) BPF diets ($p < 0.05$). Despite the main effect of diet and interaction between diet and week, weight gain at each individual week did not differ between groups.

Food intake was evaluated only during the first 7 weeks on BPF diet. During this time there was no effect of diet ($F(3,44) = 0.476$, $p = 0.71$) or interaction between diet and week ($F(3,44) = 0.874$, $p = 0.462$) on food intake.

3.4 BPF diets did not change glucose tolerance

There were no differences in baseline glucose levels between groups ($F(3,44) = 0.319$, $p = 0.812$). Similarly, the area under curve (AUC) of glucose levels throughout the sampling period was not different between groups ($F(3,44) = 0.472$, $p = 0.703$, Figure 4).

3.5 BPF consumption was reflected in plasma

Mice on control diet had undetectable plasma levels of BPF. Concentrations of BPF in plasma reflected doses of BPF in food ($F(3,44) = 2.97$, $p < 0.05$, Table 2). Planned comparisons revealed that each dose was significantly different from the two others ($p < 0.05$). Based on food intake data we estimate the mice consumed approximately 3.5 g of food per day. Given this number our estimates for the amount of BPF consumed daily are shown in Table 2.

Discussion

In general our data suggest that BPF has different actions on fat cell differentiation and weight gain than either BPA or BPS. Our results from preadipocyte cells demonstrate that at the low doses we used, BPS had the greatest effect on preadipocyte differentiation and lipid accumulation followed by BPA, with BPF having little impact, and the reverse effect (lowered mRNA), as compared with BPA and BPS. The body weight gain data also suggest

that BPF intake reduces weight gain as compared with male mice not consuming any BPF. The plasma levels and estimated intake of BPF show that *in vivo* these effects are noted at much lower levels than used in previous studies. This alone gives us cause for concern as concentrations of BPF are certainly going to increase as it is increasingly used in place of BPA.

One 3T3-L1 study similar to ours used a range of doses of BPS and BPA that started at our low dose (10 nM) and increased from there (Ahmed and Atlas 2016). In that study, levels of transcription for *Cpba*, *Ppar γ* , and *Plin* were significantly increased by BPS relative to BPA after 3T3-L1 cells experienced six days of treatment, but only at a dose 25 times higher than our high dose (1 μ M). At this same concentration (25 μ M) lipid accumulation was higher in BPA and BPS than in control treated cells, and BPS led to more lipid accumulation than BPA. In recently published study (Skledar et al. 2019) evaluation of the metabolism of BPA fluorinated analog (BPAF) on adipogenesis in 3T3L1 cells was performed at much lower exposure levels (0.1 – 10 μ M). This study showed that the level of BPA as low as 0.1 μ M significantly increased the lipid accumulation measured at day 8 into differentiation. Although not shown for lower levels of exposure, on day 4 the two higher doses of BPA (1 and 10 μ M) significantly increased transcript level of adipogenic marker *Fabp4*, 2.3-fold and 4.5-fold respectively. After 8 days of differentiation significantly higher levels of adiponectin (2.5-fold) and *Cebp- α* (1.3-fold) were detected in cells exposed to 10 μ M BPA. Using only BPA, at higher doses than we used (20 – 80 μ M), and a shorter incubation period (24 h, starting exposure at day 6 into 3T3-L1 cells differentiation) another study reported that adiponectin levels and *Adipoq* mRNA were reduced as BPA concentration was increased (Kidani et al. 2010). Moreover, in a comparison between BPF and BPA (both given at 80 μ M) adiponectin was depressed by both as compared with untreated cells, but the BPF effect was quite weak. Taken together these data show that adipocytes are sensitive to low doses of BPS, which is as potent, or even more effective than BPA in promoting differentiation and lipid accumulation. In contrast, at high doses BPA and BPF can decrease adiponectin and lipid formation while at lower doses BPF has little or no effect. When primary human preadipocytes were treated with either 10 nM or 10 μ M of BPA, BPF or BPS, several of the genes we examined were upregulated by all three bisphenols in a gene expression assay (PLIN $\frac{1}{4}$ and ADIPOQ; (Verbanck et al. 2017)). One general conclusion from the study, supported by 3T3-L1 cell work, was that higher doses of BPF are required to effect human adipocyte differentiation as compared with BPA or BPS.

There is an evidence from both *in vivo* and *in vitro* studies for BPA promoting and inducing adipogenesis (Masuno et al. 2005; Somm et al. 2009). Structural similarities between BPA, BPF, and BPS would suggest also having similar mode of action. Two transcription factors Ppar- γ and Cebp- α play a central role in coordination of expression of adipogenic genes/markers during the process of adipocyte maturation, with Ppar- γ being a master regulator of adipogenesis. However, comparative studies indicate, that at least the potential in induction of adipogenesis differ (Ahmed and Atlas, 2016; (Skledar et al. 2019). These differences may be due to the differences linked to the specific kinetic in metabolism of each of the bisphenols. A comparative study evaluating the pharmacokinetic behavior of BPA, BPS, BPF and BPAF revealed, that within environmentally relevant concentration range, BPAF glucuronide is at its highest while BPS is at its lowest rate (Karrer et al. 2018). In addition,

BPS exposure led to the highest internal concentrations of unconjugated bisphenols when equal external exposures were assumed. It is commonly considered that glucuronidase metabolites are non-active, however, Skledar et al. (2019) has reported that even glucuronidase metabolites, may affect the adipogenesis. However, our study is limited as we only evaluated the effect of bisphenols on transcript level of the adipogenic markers, without analyzing the metabolic endpoints such as insulin sensitivity, alterations in glucose transporters or secretion of adiponectin, thus we cannot assign a mechanism through which these bisphenol exerts their adipogenic effects.

The body weight data indirectly support the conclusion that BPF does not potentiate fat cell formation. Female mice exposed via their dams during pregnancy (starting on gestational day 7) and lactation to either 100 ng/kg body weight BPA or BPS were heavier than controls or females exposed to the same dose of BPF (Meng et al. 2018). The dose used was lower than ours, the timing of treatment, and sex of mice is different. Our data can also be compared to a study conducted in adult rats (Higashihara et al. 2007). However, in this case the doses we used were much lower. Based on daily food intake (3.5 g) in an adult male mouse we calculate that mice in our three dose groups consumed approximately 0.044, 0.44 or 4.4 mg of BPF per kg body weight (0.5, 5, or 50 mg of BPF per kg food). Higashihara and colleagues (2007) treated rats by gavage daily, for 4 weeks, with 20, 100, or 500 mg/kg body weight of BPF and initial weights of males were nearly 300 g. Thus, the three rat doses provided resulted in approximately 6, 30, and 150 mg of BPF intake daily. The large dose given to adult rats decreased body weights in both sexes, and females were more sensitive to BPF than males. Males that received the highest dose had significantly lower body weight when compared to controls starting at day 12 of treatment. In females, the 100 and 500 mg/kg doses decreased body weight starting on day 8 and the 20 mg/kg dose decreased body weight beginning on day 17. Food intake was reduced sporadically over the course of the study in males on the highest dose and in females on all three doses. In contrast, in our study no decrease in food intake was found, at least during the first 7 weeks of treatment. As such, as opposed to Higashigara et al (2007), we have no reason to suspect inconsistencies of treatment exposure in our study. Rats of both sexes that consumed the two highest doses displayed signs of sickness, and it is likely this caused their decreased food intake. Moreover, lowered glucose and cholesterol were found in males consuming the highest BPF dose. While in females, all doses of BPF produced decreased serum cholesterol and glucose. Finally in the highest dose groups females and males had lowered T3 and elevated T4 in plasma. We did not directly measure thyroid hormones or cholesterol in our study but baseline glucose and glucose tolerance were not affected by any doses of BPF.

The National Health and Nutritional Survey (NHANES) done in 2013–2014 (Lehmle et al. 2018) found detectable level of all three bisphenols (Mu et al. 2018) in randomly selected urine samples collected from the U.S. population, indicating that the exposure to BPA and its substitutes is almost ubiquitous. The urinary levels of bisphenols were associated mainly with age, gender and race/ethnicity. Interestingly, this and several other studies (Liao et al. 2012b; Ye et al. 2015) showed that urinary levels of BPF and BPS are lower (approximately one order of magnitude) as compared to BPA. Nevertheless, in some countries populations may have significantly higher levels of exposure to BPS as compared to the US because of country-specific environmental factors and consumers behaviors (Asimakopoulos et al.

2016; Liao et al. 2012b). However, urinary BPF and/or BPS levels are expected to be higher in the future in the US as these substituents are replacing BPA. Studies quantifying BPS in other human biological material such as serum or breast milk are scarce (Deceuninck et al. 2015; Liu et al. 2017; Thayer et al. 2016) and when detected it is usually only in few subjects. In a recent study levels of BPA and six alternatives were evaluated in serum from several elderly populations, one living near electronic waste and another “reference” population presumably not in contact with electronic waste (Song et al. 2019). All sampled individuals had detectable levels of BPA in serum, and in the group exposed to electronic waste, there was a wide range between 0.56–32 ng/mL. The next most widespread bisphenol was BPF, detected in 71% of the population. The serum levels measured ranged from not detectable to 0.58 ng/mL with an average in the electronic waste population of 0.087 ng/mL and in the reference population a slightly higher measurement of 0.17 ng/mL. Of interest BPS was only detected in 20% of the population. Both of the reported serum levels of BPF are lower than the amounts we measured in our mice. However, that is likely to be caused by our sampling bias, we collected samples during the active eating period to insure high levels in plasma.

The mechanisms by which BPF produces different effects than BPS and BPA are likely to be complex. While all three bisphenols we examined can bind to estrogen receptors, in the classic prepubertal uterine assay BPF produced little increase in weight (Yamasaki, 2002 #8). In zebrafish raking lethality of these three bisphenols on embryos and affinity for estrogen receptors produced the same order: BPA>BPF>BPS (Mu, 2018 #12). In MCR-7 cells BPF can interact with both the estrogen receptor (ER) α and with the membrane ER (Lei et al. 2018). Recent work showed that *in vivo* and *in vitro* BPF lowers testosterone production by leydig cells (Ullah et al. 2018). In cultured human fetal testes cells, BPF also reduced basal testosterone secretion and showed anti-androgenic effects. When screened for activity in a yeast activity assay BPF was shown to have antagonist effects on the androgen receptor as well as glucocorticoid receptor, and in both cases the potency of BPF was slightly higher than BPA (Roelofs et al. 2015). Recent studies have shown that, similar to BPA, BPF has the potential to interfere with the thyroid hormone (TH) signaling pathway (Zhang et al. 2018; Zhu et al. 2018). A final point is that multiple bisphenols are being detected in human tissues and the manner in which these compounds interact is nearly completely unknown (Kolatorova et al. 2018). In sum, our data and others show that more research with BPF needs to be conducted to help us decide if it is a safe alternative to BPA (Eladak et al. 2015).

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Figure 1:

Mean $\pm$ SEM expression levels of adipogenic markers in 3T3-L1 cell cultures treated with one of two doses of BPA (yellow and orange), BPF (light green and dark green), or BPS (light and dark blue). In each case the lighter bar represents the 10 nM dose and the darker bar the 1 μ M dose. The expression levels were normalized to the respective control (black) which is assigned to 1. *Significantly different from control on the same day. Significantly different from BPA and BPS in the same dose (1 μ M) on the same differentiation day. #Significantly different from other BPA dose (1 μ M). All significance levels are at least $p < 0.05$.

Figure 2:

Mean ± SEM accumulated Oil Red O in 3T3-L1 cells after incubation for 12 days with one of the bisphenols and concentrations labeled on the x-axis. *Significantly different from control and both concentrations of BPF. **Significant difference between two doses of the same bisphenol. Significantly different from both concentrations of BPA. In all cases significance is $p < 0.05$ or less.

Figure 3.

Mean $\pm$ SEM- body weight at the start of BPF diet, change after 7 weeks, and after 12 weeks of consumption. White circle/dotted line = 0 mg in diet, light grey triangle/dot-dash line = 0.5 mg/kg diet dose, dark grey square/solid line = 5 mg/kg diet dose, solid black diamond/solid line = 50 mg/kg diet concentration of BPF. *By week 12 the mice in the 0.5 mg and 50 mg/kg diet groups were significantly lighter than controls ($p < 0.05$). N=12 per group.

Figure 4.

A. Mean $\pm$ SEM glucose concentrations during the glucose tolerance test White circle/dotted line = 0 mg in diet, light grey triangle/dot-dash line = 0.5 mg/kg diet dose, dark grey square/solid line = 5 mg/kg diet dose, solid black diamond/solid line = 50 mg/kg diet concentration of BPF. B. Mean $\pm$ SEM Area under the curve. White bar = 0 mg in diet, light grey bar = 0.5 mg/kg diet dose, dark grey bar = 5 mg/kg diet dose, black bar = 50 mg/kg diet concentration of BPF. No differences were noted between the groups. N=12 male mice per group.

Table 1:

Forward (F) and Reverse (R) qPCR primers.

Target	Primer Sequence (5'→3')	Amplicon Size (bp)	Gene ID
<i>Pparγ</i>	F: GCCTGCGGAAGCCCTTTGGT	195	19016
	R: GCAGTTCCAGGGCCTGCAGC		
<i>Cebpa</i>	F: TGGACAAGAACAGCAACG	119	12606
	R: GTCAACTCCAGCACCTTC		
<i>aP2 (Fabp4)</i>	F: GGAAGCTTGTCTCCAGTGAA	200	11770
	R: GCGGTGATTTCATCGAATTC		
<i>Plin</i>	F: TTGGGGATGGCCAAAGAGAC	195	103968
	R: CTCACAAGGCTTGTTTGGC		
<i>AdipoQ</i>	F: GACGACACCAAAAGGGCTCA	213	11450
	R: GAGTGCCATCTCTGCCATCA		
<i>Cfd</i>	F: CCTGAACCCTACAAGCGATG	116	11537
	R: CAACGAGGCATTCTGGGATAG		
<i>Rpl19</i>	F: GAAGGTCAAAGGGAATGTGTCA	72	19921
	R: CCTTGTCTGCCTTCAGCTTGT		

Table 2:

Levels of BPF in plasma. Samples collected from 6 mice in each dose group. All the groups are significantly different from each other ($p < 0.05$). *Estimated BPF consumption is based on 3.5 g food consumed daily and a 40 g male mouse.

BPF (mg/kg diet)	Estimated BPF consumed daily (Yamasaki et al.)	Mean (ng/mL) BPF in Plasma	SEM
0	0	None detected	
0.5	0.044	6.20	1.01
5	0.44	43.57	6.31
50	4.4	561.33	45.33