Differences in Bisphenol A and Estrogen Levels in the Plasma and Seminal Plasma of Men With Different Degrees of Infertility

J. VITKU[1], L. SOSVOROVA[1], T. CHLUPACOVA[1], R. HAMPL[1], M. HILL[1], V. SOBOTKA[2], J. HERACEK[3,4], M. BICIKOVA[1], L. STARKA[1]

[1]Department of Steroids and Proteofactors, Institute of Endocrinology, Prague, Czech Republic,
[2]Department of Urology, Third Faculty of Medicine, Charles University in Prague, Czech Republic,
[3]Department of Urology, First Faculty of Medicine, Charles University in Prague, Czech Republic,
[4]Department of Urology, Military University Hospital Prague, Czech Republic

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Summary

The general population is potentially exposed to many chemicals that can affect the endocrine system. These substances are called endocrine disruptors (EDs), and among them bisphenol A (BPA) is one of the most widely used and well studied. Nonetheless, there are still no data on simultaneous measurements of various EDs along with steroids directly in the seminal fluid, where deleterious effects of EDs on spermatogenesis and steroidogenesis are assumed. We determined levels of BPA and 3 estrogens using LC-MS/MS in the plasma and seminal plasma of 174 men with different degrees of infertility. These men were divided according their spermiogram values into 4 groups: (1) healthy men, and (2) slightly, (3) moderate, and (4) severely infertile men. Estradiol levels differed across the groups and body fluids. Slightly infertile men have significantly higher BPA plasma and seminal plasma levels in comparison with healthy men ($p<0.05$ and $p<0.01$, respectively). Furthermore, seminal BPA, but not plasma BPA, was negatively associated with sperm concentration and total sperm count (–0.27; $p<0.001$ and –0.24; $p<0.01$, respectively). These findings point to the importance of seminal plasma in BPA research. Overall, a disruption of estrogen metabolism was observed together with a weak but significant impact of BPA on sperm count and concentration.

Key words

Bisphenol A • Estrone • Estradiol • Estriol • Seminal fluid/plasma • Blood plasma • Infertile men • LC-MS

Corresponding author

J. Vitku, Department of Steroids and Proteofactors, Institute of Endocrinology, Národní 8, 116 94, Prague 1, Czech Republic. E-mail: jvitku@endo.cz

Introduction

Bisphenol A (BPA) is a long- and well-known endocrine disruptor (ED), that still receives a considerable amount of attention from the scientific community as well as the general public, mainly because of its ubiquity in our environment and uncertainties about its effects on humans. For the most part, BPA enters the body by the ingestion of contaminated food or beverages. It leaks from polycarbonate plastics, which are used to line food and drink containers such as bottles and cans. Further minor ways of penetrating into the body are through the skin (e.g. contact with thermal receipts) (Ehrlich *et al.* 2014, Liao and Kannan 2011) or inhalation (e.g. cigarette smoke or dust) (Braun *et al.* 2011, He *et al.* 2009, Inoue *et al.* 2006, Rudel *et al.* 2003). There is still an ongoing debate whether environmental levels of BPA are harmful for the population or not.

BPA is a weak estrogen when considering its binding activities to the estrogen receptor (ER) (Welshons *et al.* 2003). On the other hand, it can act with the same potency as endogenous estradiol (E2) on the non-classical membrane estrogen receptor (Alonso-Magdalena *et al.* 2012, Quesada *et al.* 2002, Wozniak *et al.* 2005). Its mode of action, however, is much more complex. It may act through other nuclear receptors

Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

including the estrogen related receptor (Delfosse *et al.* 2014, Okada *et al.* 2008), androgen receptor (Lee *et al.* 2003, Teng *et al.* 2013), thyroid receptor (Moriyama *et al.* 2002), glucocorticoid receptor (Sargis *et al.* 2010), peroxisome proliferator activated receptor γ (PPARγ) (Pereira-Fernandes *et al.* 2013, Wang *et al.* 2010) and pregnane X receptor (Sui *et al.* 2012). An interaction of BPA with the expression and activity of steroidogenic enzymes has also been reported (Cannon *et al.* 2000, Gilibili *et al.* 2014, Hanioka *et al.* 1998, Ye *et al.* 2014, Ye *et al.* 2011, Zhang *et al.* 2011). Moreover, BPA exerts a non-monotonic dose response at low physiologically-relevant concentrations, with tissue-specific effects (for review see Wetherill *et al.* 2007).

Endogenous estrogens are thought to have an important role in the testis, because estrogen biosynthesis occurs in the testicular cells and the absence of ERs causes adverse effects on spermatogenesis as well as steroidogenesis (Akingbemi 2005). Physiological levels of E2 are essential for normal spermatogenesis; in contrast, a surplus of estrogens (together with a lack of testosterone) occurs in infertility (Pavlovich *et al.* 2001). The impact of BPA on male reproductive function is of particular interest due to its estrogenic and antiandrogenic activities, which could have potentially deleterious effects on spermatogenesis (reviewed in Hampl *et al.* 2013a,b).

Recently, our group developed a sensitive and accurate method for the simultaneous measurement of estrogens (estrone, estradiol and estriol) and BPA in human plasma and seminal fluid (Vitku *et al.* 2015). Reported levels of estrogens and BPA vary in these biological fluids in normospermic men, underlining the fact that seminal fluid is a unique environment where the effects of BPA may be expressed directly in the testis. In this study, we aimed to investigate BPA and estrogen concentrations in the plasma and seminal fluid in men with different degrees of infertility, and evaluate the potential effects of BPA on the estrogen metabolism and sperm quality.

Materials and Methods

Chemicals and reagents

Reference standards of estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d4E1) and estriol (d2E3) were purchased from Steraloids (Newport, RI, USA). Bisphenol A (BPA), deuterated BPA (d16BPA) and deuterated E2 (d3E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA) as were 99.9 % tert-butyl methyl ether (MTBE), acetone, sodium bicarbonate, sodium hydroxide and dansyl chloride. Methanol and water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of HPLC grade.

Study population and sample collection

Samples of plasma and seminal plasma were obtained from patients attending the Centre of Assisted Reproduction Pronatal (Prague, CZ). Each patient underwent a standardized ejaculate examination (spermiogram) according to the World Health Organization (WHO) 2010 criteria. In a previous study (Vitku *et al.* 2015), we dealt with the problem of ensuring that sampling equipment is not contaminated with BPA. Following the procedures detailed in that study, all steps in sample collection and processing were carried out using BPA-free glass equipment and stored in glass tubes in –20 °C until analysis. Plasma and seminal plasma samples were obtained from 174 men with different degrees of infertility. The mean age of the men was 35.97±5.64 years and BMI 27.32±3.65. Men were divided into four groups according to spermiogram values. The first group included normospermic men with a normal spermiogram (n=84); oligospermic, asthenospermic and oligoasthenospermic men were included in the second group (n=56); teratospermic, oligoasthenoteratospermic and oligoteratospermic men were placed in the third group (n=20); and the fourth group consisted of azoospermic men (n=14). We termed these groups: (1) healthy men, and (2) slightly, (3) moderately and (4) severely infertile men.

The study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all subjects participating in the project.

Determination of estrogens and BPA

We analyzed unconjugated forms of E1, E2, E3 and BPA in plasma and seminal plasma by a newly developed isotope dilution ultra high performance liquid chromatography – mass spectrometry method (Vitku *et al.* 2015). A Kinetex C18 column (100 x 3.0 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3 mm ID column; Phenomenex, Torrance, CA, USA) was used for the analysis. An Eksigent ultraLC 110 liquid chromatograph system (Redwood City, CA, USA) was coupled to an API 3200 mass spectrometer (AB Sciex, Concord, Canada) with an electrospray ionization (ESI) probe operating in positive mode.

Detailed information about the analysis procedure and validation are provided elsewhere (Vitku *et al.* 2015). Briefly, 500 µl of plasma or 1000 µl of seminal fluid was diluted by 500 µl of physiological solution (0.9 % sodium chloride) and samples were vortexed. Consequently, extraction with 2 ml of 99.9 % tert-butyl methyl ether (MTBE) for one minute was performed. The organic phase was evaporated until dryness using a vacuum concentrator (55 °C). Further, a derivatization step was carried out: a volume of 50 µl of sodium bicarbonate buffer (100 mM, pH 10.5) and 50 µl of dansyl chloride in acetone (1 mg/ml) were added to the dry residues, shortly vortexed and incubated in a heat block (60 °C) for 5 min. After removing from the heat block, samples were left to cool down to room temperature and again evaporated until dryness. Thereafter, samples were reconstituted with 300 µl of methanol, and 50 µl were transferred to the glass insert containing 50 µl of the ammonium formate in ultrapure water (10 mM) pre-pipetted. The injection volume was 50 µl.

Statistical evaluation

Before statistical analysis, the data were transformed by Box-Cox transformation due to the significant skewness, kurtosis and heteroscedasticity of most variables. Differences between groups were evaluated using one-way ANOVA followed by least square difference multiple comparisons. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations, correlations, ANOVA testing and multiple comparisons. Multiple outliers for correlations were found using NCSS 2007 (Kaysville, UT, USA).

Results

Here we report the first data on BPA exposure in a population of Czech men (Table 1). The groups of men did not significantly differ from each other in age and BMI. We detected BPA, E2, E1 and E3 in 87 %, 94 %, 100 % and 62 % of plasma samples, respectively, and 92 %, 84 %, 90 % and 97 % of seminal samples, respectively. In the rest of the samples, levels were under the lower limit of quantification.

Table 1. Comparisons of age, BMI, BPA (pg/ml) and estrogen levels (pg/ml) in groups of men with different degrees of infertility.

		Group 1 (n=84)	Group 2 (n=56)	Group 3 (n=20)	Group 4 (n=14)	p-value	Multiple comparisons
	Age	36 (34.9; 36.7)	35.8 (34.5; 37.2)	35.7 (33.4; 37.9)	35.2 (32.5; 37.9)	0.959	
	BMI	27.4 (26.2;28.5)	27.5 (26.2;28.7)	26.6 (24.9; 28.2)	26.2 (24.9; 28.2)	0.451	
Plasma	BPA	47 (26;81)	137 (75; 239)	114 (42; 270)	33 (6;125)	**0.036**	1<2
	E2	22 (18;26)	18 (15;23)	17 (11;24)	7 (3;12)	**0.002**	1,2,3>4
	E1	24 (20;29)	23 (18;28)	21 (15;29)	17 (10;26)	0.513	
	E3	19 (11;31)	20 (10;36)	19 (5;53)	13 (3;36)	0.933	
Seminal fluid	BPA	66 (44;94)	144 (98;205)	132 (69;228)	179 (84;330)	**0.009**	1<2,4
	E2	2 (1;3)	4 (3;6)	9 (5;15)	7 (3;14)	**0.002**	1<3,4; 2<3
	E1	4 (3;5)	5 (4;7)	9 (6;13)	6 (3;9)	**0.008**	1,2<3
	E3	43 (30;59)	31 (19;47)	34 (16;63)	83 (40;154)	0.129	2<4

Data are shown as means and 95.0 percent confidence intervals (in parentheses) for each group, with levels of significance for the ANOVA and multiple comparisons provided. Group 1 = normospermic men; Group 2 = oligospermic/asthenospermic/oligoasthenospermic men; Group 3 = teratospermic/oligoteratospermic/oligoasthenoteratospermic men; Group 4 = azoospermic men.

Comparisons of plasma and seminal BPA and estrogen levels in men with different degrees of infertility

Generally, BPA levels in seminal fluid were slightly higher than those in plasma, except for in the 4th group where seminal fluid BPA levels were nearly three times greater (Table 1). Seminal BPA concentrations were found to be higher in all groups of men with various degrees of infertility in comparison with normospermic men. Plasma BPA levels were significantly higher in the group of slightly infertile men compared to healthy men. Concentrations of plasma E2 decreased from the first to the fourth group, while seminal E2 levels increased. Mean plasma levels of E2 varied from 7 to 22 pg/ml among the groups, in comparison with 2-7 pg/ml mean E2 in semen. Plasma and seminal fluid E1 levels showed similar results as for E2. Concentrations of E3 in seminal fluid were significantly higher than E3 plasma levels in all groups, and did not differ across the groups. A graphical representation of the differences in BPA and E2 levels in both fluids across the groups is shown in Figure 1.

Correlation matrix between BPA, estrogens and spermiogram parameters

Pearson´s correlation coefficients between all parameters are provided in Table 2. Although plasma and seminal BPA levels correlate with each other, only BPA in seminal fluid was negatively associated with sperm concentration and total sperm count. This indicates the importance of seminal fluid in research on the effects of BPA. Another finding that is of interest is the positive association between BPA and E2 in both body fluids.

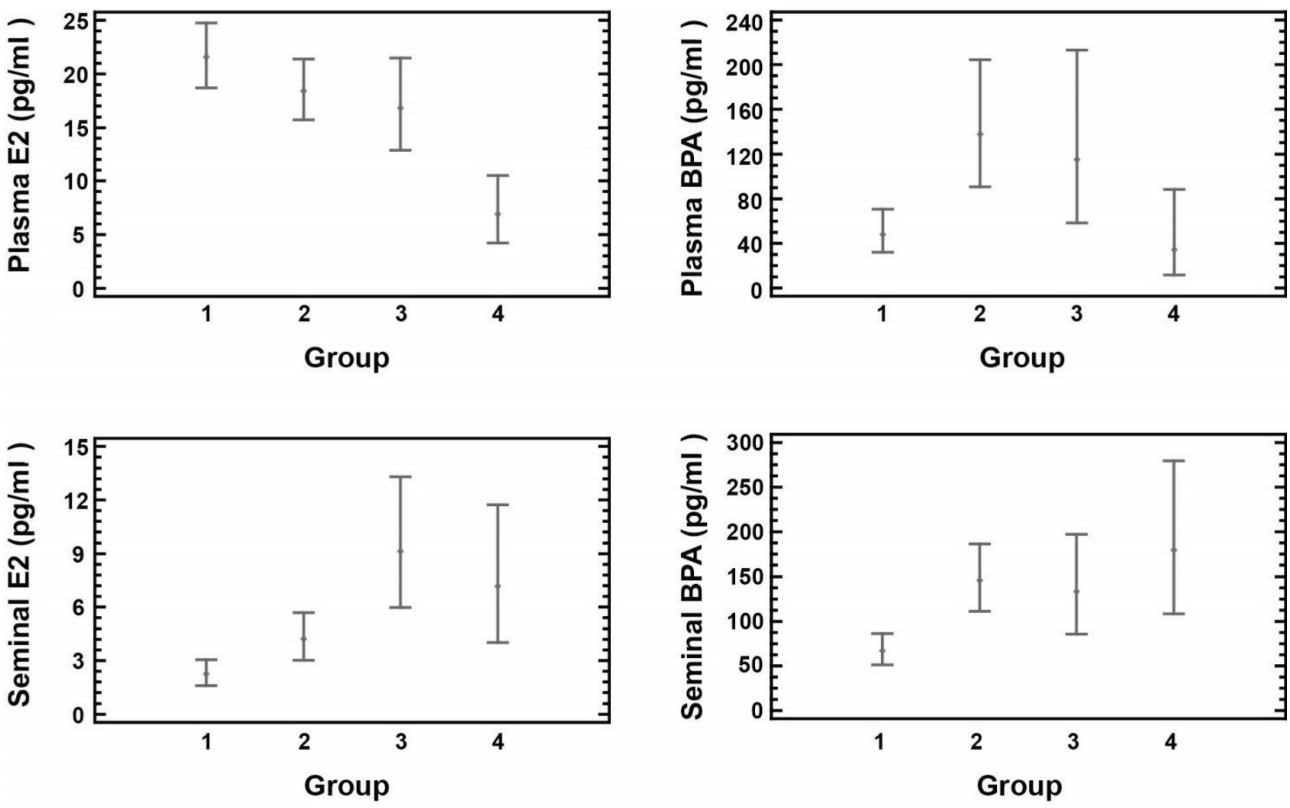

Fig. 1. Differences in bisphenol A (BPA) and estradiol (E2) levels in plasma and seminal plasma among the 4 groups of men. Group 1 = normospermic men (n=84); Group 2 = oligospermic/asthenospermic/oligoasthenospermic men (n=56); Group 3 = teratospermic/ oligoteratospermic/oligoasthenoteratospermic men (n=20); Group 4= azoospermic men (n=14).

Discussion

To the best of our knowledge, this is the first study reporting levels of EDs and steroids directly in seminal fluid. We measured unconjugated forms of estrogens as well as BPA, thought to be the active forms, and found that the levels differ in seminal fluid and plasma. This indicates that seminal fluid is a unique milieu that deserves further investigation.

Experimental studies in adult rodents have provided evidence that exposure to BPA affects sperm quality and production (reviewed in Peretz *et al.* 2014). However, few studies have reported the impact of BPA on sperm quality in adult men. To our knowledge, six studies have dealt with this problem, all measuring BPA in urine, and with divergent results (Goldstone *et al.* 2014, Knez *et al.* 2014, Lassen *et al.* 2014, Li *et al.* 2011, Meeker *et al.* 2010b, Mendiola *et al.* 2010). Our finding of decreasing sperm concentrations and counts in association with increasing seminal BPA (Table 2) is in agreement with three of the studies (Knez *et al.* 2014, Li *et al.* 2011, Meeker *et al.* 2010b). In contrast, the other studies did not find any association between these sperm parameters and urinary BPA (Goldstone *et al.* 2014, Mendiola *et al.* 2010). However, Lassen *et al.* (2014) reported a significant inverse association between BPA excretion and progressive motility. Still, the outcomes vary across studies, apparently due to the difficulties in evaluating the effects of EDs when organisms are exposed more than one at a time.

Table 2. Pearson´s correlation coefficients among levels of plasma and seminal BPA and estrogens, and spermiogram parameters. Correlation coefficients are provided in the first row of each variable, p-values are in the second row. If significant, coefficients and p-values are highlighted in bold.

	Plasma E2	**Plasma E1**	**Plasma E3**	**Plasma BPA**	**Seminal E2**	**Seminal E1**	**Seminal E3**	**Seminal BPA**	**Concentration**	**Total count**	**Motility**	**Progressive motility**	**Morphology**
Plasma E2	1.000												
Plasma E1	**0.372** **0.000**	1.000											
Plasma E3	**0.366** **0.001**	0.173 0.144	1.000										
Plasma BPA	**0.363** **0.000**	-0.146 0.120	0.192 0.103	1.000									
Seminal E2	-0.065 0.498	**-0.236** **0.012**	0.190 0.110	0.076 0.378	1.000								
Seminal E1	0.035 0.714	-0.004 0.967	0.124 0.299	0.058 0.499	**0.181** **0.023**	1.000							
Seminal E3	**0.229** **0.016**	-0.059 0.538	**0.384** **0.001**	0.106 0.218	**0.192** **0.016**	**0.158** **0.047**	1.000						
Seminal BPA	0.082 0.395	**-0.257** **0.006**	0.185 0.120	**0.338** **0.000**	**0.318** **0.000**	0.061 0.450	0.156 0.051	1.000					
Concentration	-0.006 0.952	0.053 0.575	-0.003 0.983	-0.160 0.057	-0.146 0.066	-0.098 0.222	0.063 0.429	**-0.271** **0.001**	1.000				
Total count	-0.059 0.536	0.077 0.412	-0.022 0.852	-0.163 0.052	**-0.164** **0.039**	-0.106 0.184	0.010 0.898	**-0.236** **0.003**	**0.957** **0.000**	1.000			
Motility	**0.293** **0.002**	0.164 0.081	**0.268** **0.022**	0.065 0.439	**-0.248** **0.002**	**-0.187** **0.019**	0.021 0.796	-0.115 0.151	**0.513** **0.000**	**0.506** **0.000**	1.000		
Progressive motility	**0.291** **0.002**	0.172 0.066	**0.231** **0.049**	0.012 0.886	**-0.244** **0.002**	**-0.225** **0.005**	0.027 0.732	-0.129 0.106	**0.546** **0.000**	**0.550** **0.000**	**0.958** **0.000**	1.000	
Morphology	0.096 0.310	-0.010 0.918	0.133 0.264	-0.103 0.223	**-0.203** **0.010**	**-0.185** **0.020**	0.013 0.870	-0.124 0.119	**0.595** **0.000**	**0.561** **0.000**	**0.722** **0.000**	**0.741** **0.000**	1.000

The levels of BPA in the plasma of group 4 are similar to those of group 1, i.e. of normal fertile men. One possible reason may be that the severe infertility in men of group 4 is caused by other factors than the effects of hormones and other constituents of seminal fluid, e.g. genetic or anatomic causes or the result of infections.

Although the body of ED research is continuously expanding, there still exist uncertainties in the process of BPA degradation in the body. It has generally been thought that BPA is rapidly metabolized in the liver and excreted in the urine within hours (Volkel *et al.* 2002, 2005). On the other hand, a recent study showed that during fasting BPA levels did not decline rapidly, suggesting a substantial non-food-related exposure or accumulation in tissues (Stahlhut *et al.* 2009). This is why we decided to study the relationships between EDs and sperm quality in seminal fluid, with its closer proximity to sperm production. According to our previous study, seminal and plasma BPA levels in normospermic men did not correlate with each other, indicating that their distribution or metabolism are different in these body fluids (Vitku *et al.* 2015). Furthermore, BPA competes with sex steroids for human plasma SHBG (Dechaud *et al.* 1999), suggesting that the bioavailability and half-time in blood could be affected. Further studies on the persistence of BPA in semen and other body fluids during chronic exposure are needed.

Studies that have investigated associations between BPA and steroidogenesis are divergent as well. Only a few of them have investigated the impact of BPA on estrogen levels and estrogen metabolism. Urinary BPA was reported to be positively associated with plasma E2 in a group of young men (Lassen *et al.* 2014). Another study showed no association with E2 levels (Galloway *et al.* 2010) and another reported an inverse relationship (Meeker *et al.* 2010a).

Our results show that the levels of E2 differ across the groups and body fluids. Only 10-25 % of the E2 in circulation in men is synthesized in the testis. Aromatase activity and estrogen biosynthesis in men occur mainly in adipose tissue (Levine *et al.* 1997). In our study, the increases of E2 and E1 in the seminal plasma of increasingly infertile men and the opposite trends in plasma raise some considerations. One explanation is that peripheral estrogens penetrate more easily through the blood-testis-barrier to the testis. Alternatively, estrogens originating in the testis may have a harder time accessing the periphery. Other possibilities include the increased expression or activity of aromatase in the testis and/or decreased expression or activity in the adipose tissue. The metabolism of E2 could be also protracted by the interaction of BPA with enzymes involved in steroid conjugation such as estrogen glucuronidase or sulfotransferase. This explanation is in accordance with the *in vitro* study of Zhang *et al.* (2011), who reported that BPA suppressed E2 catabolism in H295R cells but without altering aromatase activity. On the other hand, studies from *in vitro* and *in vivo* experiments focused on the impact of BPA on aromatase activity have yielded conflicting results (Castro *et al.* 2013, Ehrlich *et al.* 2013, Rajakumar *et al.* 2015, Shanthanagouda *et al.* 2014).

In conclusion, the results of our study show an inverse association between seminal BPA and sperm count and concentration. The correlation coefficients were relatively weak ($r=-0.24$ and $r=-0.27$, respectively) suggesting that BPA slightly, but significantly, contributes to the final state of sperm quality, together with other factors. Moreover, a disruption of estrogen metabolism was observed, but the mechanism of action remains to be elucidated.

Conflict of Interest

There is no conflict of interest.

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