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Short communication

Serum unconjugated bisphenol A concentrations in men may influence embryo quality indicators during *in vitro* fertilization☆

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ABSTRACT

Here we assess bisphenol A (BPA) in couples undergoing *in vitro* fertilization (IVF) and indicators of embryo quality; embryo cell number (ECN) and embryo fragmentation score (EFS). Twenty-seven couples provided serum on the day of oocyte retrieval. Unconjugated BPA was measured by HPLC with Coularray detection. Odds ratios (OR) were generated using ordinal logistic regression including female and male BPA concentrations, age and race, and day of embryo transfer for ECN. Inverse associations are suggested for male BPA with ECN (OR = 0.70, $P = 0.069$), and EFS (OR = 0.54, $P = 0.009$), but not for women. Male BPA exposure may affect embryo quality during IVF.

1. Introduction

Growing evidence indicates that bisphenol A (BPA), a widely used plastic monomer, is a reproductive toxicant of public health importance (NTP, 2008). However, this issue remains controversial (Goodman et al., 2009; Vandenberg et al., 2009). BPA has been shown to disrupt oocyte nuclear competence during murine folliculogenesis (Hunt et al., 2003), with deleterious effects on nuclear maturation including increased mitotic spindle defects and meiotic arrest. BPA also binds to 'classical' nuclear estrogen receptors (Richter et al., 2007; Wetherill et al., 2007), and to 'non-classical' membrane-associated estrogen receptors (Luconi et al., 1999; Wozniak

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et al., 2005). However, the clinical implications of BPA estrogen receptor binding remain unclear (Luconi et al., 2001).

The potential public health significance of BPA-associated reproductive toxicity is underscored by its detection in 93% of the U.S. population (Calafat et al., 2008), as well as in human reproductive fluids (Inoue et al., 2002; Kaddar et al., 2009). In this preliminary study, we augment our prior work (Fujimoto et al., 2011b) describing an inverse association between female BPA concentrations and oocyte fertilization rates during *in vitro* fertilization (IVF). The current study describes associations between BPA concentrations and embryo quality indicators among a subset of couples for whom embryos were generated during IVF.

2. Materials and methods

The current analysis comprises a subsample of 27 couples participating in the previously described prospective cohort Study of Metals and Assisted Reproductive Technologies (SMART) (Bloom et al., 2011). This dataset includes only those members of the SMART cohort for whom embryos were generated during IVF, and for whom a sufficient volume of serum was available to complete BPA analysis in both the female patient and her male partner. Informed consent was obtained from all participants, and the study protocol was approved by the University of California at San Francisco Committee for Human Research. Women underwent gonadotropin-induced ovarian stimulation according to clinic protocols, and oocytes were retrieved 36 h after administration of human chorionic gonadotropin. On the day of oocyte retrieval, fasting and non-fasting blood specimens were collected from female patients and male partners, respectively, into SST serum separator Vacutainer® tubes (Becton Dickinson and Co., Franklin Lakes, NJ) let sit for 20–30 min, and centrifuged at 700 × *g* for 10 min. Serum was aliquoted into 1.8 ml polypropylene cryovials and frozen at −80 °C. Oocytes were fertilized by conventional insemination or by intracytoplasmic sperm injection (ICSI) using fresh sperm from male partners. Zygotes were identified by the appearance of two pronuclei 16–18 h after insemination. Embryo cell number (ECN) was assessed on the day of embryo transfer as the number of blastomeres present. Embryo fragmentation score (EFS) was assessed on the day of transfer (usually 48 h post-fertilization) as: Grade 1, 0% fragmentation; Grade 2, 1–10%; Grade 3, 11–25%; Grade 4, 26–50%; and Grade 5, ≥51%.

Serum specimens were shipped on dry ice to the Endocrine Disruptors Laboratory at the University of Missouri (Columbia, MO). Specimens (1–2 ml) were twice extracted with methyl tert-butyl ether, recombined, dried down under nitrogen, and then reconstituted in methanol. Unconjugated BPA concentrations in sample extracts were determined by high performance liquid chromatography (HPLC), using known standards, with an ESA Coularray 5600 detector (ESA Inc., Chelmsford, MA). Separation was performed on a reverse-phase 250 mm Prodigy C18 column (Phenomenex, Torrance, CA), with a mobile phase of 36:24:40 acetonitrile:methanol:0.05 M sodium acetate buffer (pH 4.8), and with the Coularray cell potentials set at 325, 400, 720 and 875 mV. Empty serum collection tubes and laboratory diluent and extraction blanks did not contain detectable BPA. Recovery averaged 89% and the method limit of detection (LOD) was 0.3 ng/ml. To preclude the introduction of statistical bias (Schisterman et al., 2006), machine-read values were reported without censoring values below the LOD.

Adjusted associations between log-transformed serum BPA concentrations, EFS and ECN were estimated using ordinal logistic regression models (McCullagh, 1980). This approach estimates the log odds of an outcome falling into the *k*th ($k = 1, 2, 3$) category or lower across $k - 1$ ordered response categories. In effect, two traditional binary logistic regression models are 'collapsed' into a single model under the assumption of proportional odds. Model coefficients and 95% confidence intervals (CI) were exponentiated to provide adjusted odds ratios (aOR) and their 95% CIs. Generalized estimating equations (GEE) were used to provide robust standard error estimates as outcomes were correlated within couple (Zeger and Liang, 1986). Based on review of the literature (Calafat et al., 2008) and use of directed acyclic graphs (Greenland et al., 1999), patient and partner age (years) and race ("not-Asian"/"Asian") were *a priori* selected as confounding variables for inclusion; day of embryo transfer was included in the model for ECN. Statistical significance was defined as $P < 0.05$ for a 2-tailed test. SAS version 9.1.3 (SAS Institute, Cary, NC) was used for statistical analysis.

3. Results

Demographics for the SMART cohort have been previously described (Bloom et al., 2011). Among female and male SMART participants producing embryos, there are no meaningful differences in serum BPA concentration, age, or race ($P \geq 0.15$ by Mann–Whitney *U*-test or Fisher's Exact test as appropriate) between subjects included in this analysis and those excluded (data not shown). The current subsample produced a median of 6.0 embryos (range 1.0–14.0) per couple, with median ECN equal to 6.4 (range 3.0–9.3), and median EFS equal to 2.3 (range 1.2–4.0). Women (median 35 years; range 31–44) are somewhat younger than men (median 38 years; range 31–48), are more likely to report an Asian race (33.3% vs. 18.5%), and demonstrate higher BPA concentrations (median 3.3 ng/ml; range 0.0–67.4; 85.2% >LOD for women vs. 0.48 ng/ml, range 0.0–22.7; 51.9% >LOD for men; $P = 0.006$ using paired Student *T*-test for log BPA).

A total of 186 and 184 embryos are included in the ordinal logistic regression models for ECN and EFS, respectively. There is no association for BPA measured in women with ECN (aOR 1.06, 95%CI 0.74, 1.50; $P = 0.76$), or with EFS (aOR 1.02, 95%CI 0.68, 1.52; $P = 0.92$), adjusted for male BPA as well as for the age and race of each partner, and day of transfer for ECN. However, in the same models a 30% decrease in the adjusted odds for a higher ECN is suggested (aOR 0.70, 95%CI 0.48, 1.03, $P = 0.069$), and a 46% decrease in the adjusted odds for higher EFS is detected (aOR 0.54, 95%CI0.34, 0.86; $P = 0.009$) for each log-unit increase in male BPA. In the ECN model, Asian race among women is a positive predictor (aOR 5.68, 95% 1.34, 25.62; $P = 0.02$); however, not among men (aOR 0.31, 95%CI 0.07, 1.40; $P = 0.127$). Suggestive increases and decreases in ECN are also detected for each year increase in age among women (aOR

1.13, 95%CI 0.98, 1.30; $P = 0.089$) and among men (aOR 0.90, 95%CI 0.81–1.00; $P = 0.060$), respectively. No additional effects are suggested or detected for EFS.

Figs. 1 and 2 describe the regression models between ECN or EFS, and log male serum BPA after adjustment for the aforementioned covariates; each figure describes two binary logistic regression models which are collapsed to provide the overall ordinal logistic regressions for ECN and EFS. Fig. 1 shows increasing male BPA associated with a decreasing probability for a couple's embryos to have an ECN of 6–12 cells, relative to a reference category of ECN equal to 1–5 cells (solid red line of best fit), as well as a decreasing probability for a couple's embryos to have an ECN of 8–12 cells relative to a reference category equal to 1–7 cells (broken green line of best fit). Fig. 2 shows increasing male BPA associated with a decreasing probability for a couple's embryos to have an EFS of 2–5, relative to a reference category equal to 1 (solid red line of best fit), as well as a decreasing probability for a couple's embryos to have an EFS of 3–5 relative to a reference category equal to 1–2 (broken green line of best fit).

4. Discussion

The effects on embryo quality indicated by this study for only paternal serum BPA concentrations were unanticipated *a priori*. It is tempting to contemplate that the inverse association suggested for early embryo cleavage rate, may result from sperm integrity disruption. Whereas the nucleic acid sequence of sperm DNA does not appear to be directly altered by BPA (Bennetts et al., 2008), other structural changes have been reported in BPA-exposed sperm (Toyama et al., 2004), and structural changes in the male haplosome induce alterations in embryo development (Sakkas and Alvarez, 2010). Greater embryo fragmentation is generally associated with poorer prognosis (Fujimoto et al., 2011a). The unexpected detection of a 'protective' effect for male BPA on embryo fragmentation as assessed by EFS might reflect incomplete capture of a non-monotonic dose-response curve (Welshons et al., 2003), a consequence of the limited sample size. That is, increased fragmentation may occur at lower and higher doses than those measured in our study, with exposures in the study sample representing the nadir or peak of a non-monotonic or 'U-shaped' dose-response curve (Calabrese and Baldwin, 2001); although at the current time this explanation is entirely speculative. Somewhat analogous observations were reported for a mouse model in which embryonic progression from the 2 to 8-cell stage and beyond was advanced by treatment with up to 0.7 ng/ml BPA, whereas 23 ng/ml BPA inhibited progression (Takai et al., 2000); 0.7 ng/ml is within the range for unconjugated BPA in serum found here and by others (Vandenberg et al., 2010). Alternatively, these results may reflect a true, heretofore unexplained, biologic phenomenon.

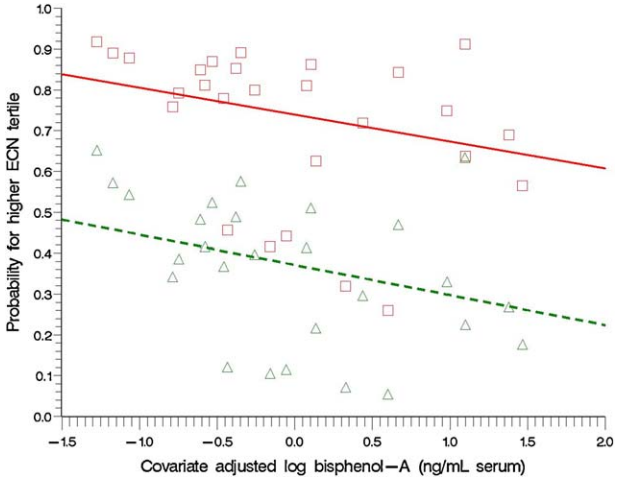

Fig. 1 – Associations between covariate-adjusted unconjugated serum BPA in 27 men participating in the Study of Metals and Assisted Reproductive Technologies (SMART) and embryo cell number (ECN). *Note*: Solid red line describes probability for a couple's embryos to have an ECN of 6–12 cells, relative to a reference category of ECN equal to 1–5 cells; broken green line describes probability for a couple's embryos to have an ECN of 8–12 cells relative to a reference category equal to 1–7 cells; probabilities estimated by ordinal logistic regression models employing generalized estimating equations (GEE) for 186 embryos, and adjusted for female unconjugated serum BPA, female and male age, female and male race, and day of embryo transfer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

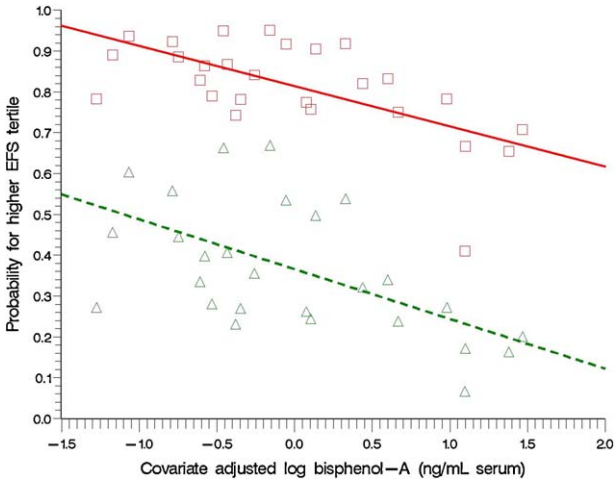

Fig. 2 – Associations between covariate-adjusted unconjugated serum BPA in 27 men participating in the Study of Metals and Assisted Reproductive Technologies (SMART) and embryo fragmentation score (EFS). *Note*: Solid red line describes probability for a couple's embryos to have an EFS of 2–5, relative to a reference category of EFS equal to 1; broken green line describes probability for a couple's embryos to have an EFS of 3–5 relative to a reference category equal to 1–2; probabilities estimated by ordinal logistic regression models employing generalized estimating equations (GEE) for 184 embryos, and adjusted for female unconjugated serum BPA, female and male age, and female and male race. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Curiously, we do not detect any effect between female serum BPA concentrations and embryo cleavage rate or fragmentation. A growing body of literature describes BPA-associated disruptions of oocyte meiosis in murine models (Hunt et al., 2009) leading to aneuploidy, increased embryo fragmentation, and arrested development (Magli et al., 2007). We thus hypothesized *a priori* that embryo cleavage would be negatively influenced, and embryo fragmentation positively influenced by female BPA concentrations (Can et al., 2005; Eichenlaub-Ritter et al., 2008; Lenie et al., 2008) given the primary role ascribed to the oocyte (Fujimoto et al., 2011a).

The small size of this study precludes conclusive evaluation of associations between BPA and embryo quality indicators, and these results should thus be considered preliminary. Participants considered during this study comprise a 'sub-sample' of the larger SMART cohort; however, comparisons between SMART participants included and excluded from this sub-study, and generating embryos, indicate no difference. Due to the limited sample size, we were unable to consider or incorporate infertility diagnosis, stimulation protocol type, or insemination approach into our study; statistical estimates become instable and regression model do not converge upon further data stratification. These clinical factors may be important modifiers of the effects reported herein (Barroso et al., 2009) and will be considered as such in a future, larger study. It is also possible that our study results are influenced by uncontrolled confounding by other unconsidered and possibly important environmental reproductive toxicants. These results might also be caused in part by laboratory noise, given the substantial proportion of male BPA values below the LOD. However, there is little change in BPA effect estimates using various common procedures to censor values below the LOD (Hornung and Reed, 1990) (data not shown). Moreover, follicular or seminal fluid BPA measurements tracked to individual embryos would provide more accurate correlations. Despite the aforementioned limitations, this study is strengthened by our use of a biomarker for the biologically active BPA fraction (Matthews et al., 2001), and furthermore by our collective assessment of maternal and paternal BPA concentrations.

5. Conclusions

In summary, we describe inverse associations between serum unconjugated BPA concentrations measured in the male partners of women undergoing IVF, and embryo quality indicators. To our knowledge, these data represent the first evidence of male BPA exposure effects on human embryo quality and suggest a role for sperm quality on early embryo development. The limited sample size and scope of this study make these results preliminary; however, the provocative nature of these results warrants confirmation.

Conflict of interest

Nothing declared.

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