Phthalates and bisphenol do not accumulate in human follicular fluid

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Abstract

Objective To determine if phthalates and bisphenol A accumulate in human follicular fluid after brief exposure to medical plastics during an IVF cycle

Study design Prospective collection of follicular fluid from five infertile women undergoing oocyte retrieval at a University IVF laboratory and analysis of Phthalate & Bisphenol A levels.

Results All phthalate levels were detected at levels less than 15 ng/mL and Bisphenol A levels were undetectable in all five samples. The concentrations of phthalates are 200–1000 fold less than the minimum levels reported to cause

reproductive toxicity in vitro to cumulus-oocyte complexes of laboratory animals.

Conclusions In reproductive age women undergoing infertility treatments there is little transfer or accumulation of phthalates, phthalate metabolites or bisphenol A into the microenvironment of the human preovulatory oocyte and the levels are not clinically significant. Further investigation of phthalate and bisphenol A accumulation in vivo in human follicular fluid may not be productive.

Keywords Phthalates · Bisphenol A · Follicular fluid · Reproductive toxins

Capsule Follicular fluids obtained from 5 infertility patients previously exposed to medical plastics and undergoing oocyte retrieval demonstrate little transfer or accumulation of phthalates, phthalates metabolites or bisphenol A into the follicular microenvironment.

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Introduction

Phthalates and bisphenol A, present in household items and medical supplies, are under intense scrutiny as environmental reproductive toxins. In men, phthalate exposure has been linked to abnormal semen parameters and infertility [1, 2]. In laboratory animals, phthalates and bisphenol A disrupt oocyte maturation [3–13], impair intraovarian steroidogenesis [14–19], and disrupt development of female reproductive organs [20–24]. In women, phthalates and bisphenol A have been detected in blood, urine and breast milk [25–28]; however, the levels in these studies are substantially less than the levels required to induce the levels of ovarian dysfunction observed in laboratory animals. For these disruptors to impact reproductive performance in women, there would need to be preferential accumulation of these substances in the ovarian microenvironment.

To test the hypotheses that accumulation of phthalates and bisphenol A occurs in human ovaries, we harvested follicular fluids from individual infertile women residing in an industrial region of the northeast United States and

undergoing oocyte retrieval as part of their infertility treatment. Because of this treatment, all of the women had recently been exposed to medical plastics. That exposure included repeated use of plastic syringes for injecting gonadotropins, GnRH agonists, and progesterone; infusion of IV fluids stored in plastic bags through plastic tubing prior to IVF; aspiration of oocytes through plastic tubing during collection, exposure to anesthetic tubing at the time of retrieval, and repeated exposure to vaginal ultrasound probes covered with plastic sheaths over weeks or months. These fluids were then analyzed for concentrations of phthalates, phthalate metabolites and bisphenol A. Our purpose was to determine if the ovarian micro environmental accumulation of these disruptors was sufficient, based on comparisons to laboratory animals, to impair human oocyte maturation and potentially be a factor in the impaired fertility of these women.

Materials & methods

IRB approval was obtained prior to collection in September 2008 of discarded follicular fluid from five randomly selected women undergoing in vitro fertilization cycles. To be included, fluids would have been obtained from cases representative of our IVF clinic population. This means they must have been infertile for more than one year, been between 21 and 43 years of age, have had a qualifying day 3 FSH, and have had a normal uterine cavity. They must have undergone hyperstimulation with FSH and GnRH agonists with recovery of oocytes, and undergone embryo transfer with viable embryos. Rocket SX 17 gauge aspiration needles (Rocket Medical, Hingham, MA) were used to collect the follicular fluid into Falcon 14 mL polystyrene round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ) containing 2–4 mL of Quinn's Advantage Media with Hepes (Sage In-Vitro Fertilization Inc, Trumbull, CT). The amount of media in the tube was noted and tabulated prior to collection of the follicular fluid. After oocytes were recovered from the follicular fluid and media in the in vitro fertilization laboratory, the follicular fluid and media were placed in inert 120 mL Eagle pitcher amber jars (Anachemia, Miami, OK). Collection stopped at the end of the retrieval or when the total fluid collected exceeded 100 mL. The jars were placed in the freezer and sent overnight to AXYS Analytical Services Ltd. (Sidney, British Columbia) for analysis.

The samples were extracted in accordance with AXYS method MLA-059 "Analytical Procedure for the Analysis of Bisphenol A and Phthalate Ester Metabolites in Urine" based on previously described methods [29]. Analysis of target analytes was performed using Alliance 2795 HT HPLC system (Waters Inc, Milford, MA) coupled to ESI-

MS/MS triple quad MS, Micromass Quattro Ultima (Waters Inc, Milford, MA) running MassLynx 4.1 software. HPLC involved a 20 μ L injection onto a Waters Xterra C18MS column (10 cm, 2.1 mm i.d., 3.5 μ m particle with C18 guard column) for Bisphenol A and a 20 μ L injection onto a Sunfire C18 analytical column (3.5 μ m, 4.6 mm x 30 mm) for phthalate metabolites. The flow rate for both bisphenol A and phthalate esters was 0.15 to 0.20 ng/mL. The LC mobile phase for phthalate metabolites consisted of Solvent A, 0.1 % AcOH in H₂O and solvent B, 0.1 % AcOH in CH₃CN. The LC gradient was from 60 % solvent A, 40 % solvent B to 100 % solvent B. The LC mobile phase for bisphenol A consisted of Solvent A, H₂O adjusted to pH with NH₄OH and solvent B, CH₃CN. The LC gradient was from 90 % solvent A, 10 % solvent B to 100 % solvent B. The mass spectrometer was operated at unit mass resolution in the multiple reaction monitoring (MRM) mode. One MRM transition was monitored for each target compound and internal standard.

Final concentrations of Monomethyl phthalate (m-MP), Monoethyl phthalate (m-EP), Mono-n-butyl phthalate (m-BuP), Monobenzyl phthalate (m-BzP), Mono-2-ethylhexyl phthalate (m-EHP), Mono-(2-ethyl-5-oxohexyl) phthalate (m-EOHP), Mono-(2-ethyl-5-hydroxyhexyl) phthalate (m-EHHP) and bisphenol A were determined by isotope dilution internal standard quantification procedure. For all target compounds, linear equations were determined from a 6 point calibration series for bisphenol A and a 7 point calibration series for phthalate metabolites with 1/X weighting fit. Reporting limits are the greater of the lowest calibration standard concentration equivalent or the sample detection limit (SDL). SDLs are determined by converting the area equivalents to 3x the height of the chromatographic noise to a concentration. The concentration of 4-methylumbelliferone is used to monitor the efficiency of deconjugation by β -glucuronidase. The influence of sample matrix on recoveries was investigated by spiking known amounts of authentic bisphenol A (1.09 ng/mL) and phthalate metabolites (10 ng/mL) into a 1 mL follicular fluid sample and analyzing. The dilutional effect of the media was taken into account during the calculations and accounted for in the final concentrations which are based on the amount of bisphenol A and phthalates in the follicular fluid alone.

Results

The average specimen volume which included follicular fluid and collection media from all 5 patients was 77.4 $\pm$ 21.8 mL (range 60–109.5 mL) and the average follicular fluid volume within each specimen was 47.8 $\pm$ 20.9 mL (range 22–80 mL). Bisphenol A, phthalates and phthalate metabolite levels were successfully analyzed from the

follicular fluid of the five patients. Bisphenol A was undetectable in all five samples. Spiking of samples 1, 2, 3 and 5 with d6 Bisphenol A, performed as a control for detection, averaged 99.8 % recovery (range 94.3 to 112 %).

Monoethyl phthalate (m-EtP), Mono-n-butyl phthalate (m-BuP) and Mono-2-ethylhexyl phthalate (ng/mL) (m-EHP) were detected in all five patients. Monoethyl phthalate (m-EtP) levels averaged 3.19 ng/mL (range 1.49–8.39 ng/mL), Mono-n-butyl phthalate (m-BuP) levels averaged 1.62 ng/mL (range 1.08–2.63 ng/mL) and Mono-2-ethylhexyl phthalate (m-EHP) levels averaged 9.34 ng/mL (range 5.96–14.1 ng/mL). Monomethyl phthalate (mMP) was detected only in the follicular fluid of patient 1 (1.37 ng/mL) and 5 (1.01 ng/mL) (Table 1). Mono-(2-ethyl-5-hydroxyhexyl) phthalate (m-EHHP) was only detected in patient 4 at 1.43 ng/mL. Monobenzyl phthalate (m-BzP) and Mono-(2-ethyl-5-oxohexyl) phthalate (m-EOHP) levels were not detectable in any of the five patients. Spiking of sample 5 with all seven phthalate & phthalate metabolites, performed as a control for detection, averaged a 92.4 % recovery rate (range 85.9 to 103 % recovery). Spiking of sample 5 with all seven ¹³C-labeled phthalate & phthalate metabolites averaged a 92.0 % recovery rate (range 82.2 to 103 % recovery).

Comment

This is the first study to determine if phthalates accumulate in the ovarian follicular microenvironment. All phthalate levels were below 15 ng/mL. These levels are comparable to previously published serum levels in women [26] in which the mean levels of phthalates ranged 0.31 to 5.9 ng/mL and their metabolites 0.77 to 1.8 ng/mL. This suggests

that phthalates and their metabolites are neither preferentially concentrated nor excluded from follicular fluid, and that phthalate levels in follicular fluid are very low.

The levels of phthalates in human follicular fluid are several orders of magnitude below minimum levels known to cause reproductive toxicity to the oocytes of laboratory animals. Conversion of standard units demonstrates that one ng/mL of Mono-2-ethylhexyl phthalate (MEHP) or Monoethyl phthalate (m-EP) corresponds with a 3.6 or 5.1 (nM) nanomolar concentration, respectively. The highest detected phthalate concentration in our study was 14.1 ng/mL of MEHP (51 nM). Multiple animal studies indicate that much higher levels of phthalates are required to have a detrimental effect on oocytes. In 2003, Anas [4] demonstrated the lowest concentrations of MEHP that had a detrimental effect on in vitro maturation of cumulus-oocyte complexes and denuded oocytes, were 25 and 10 μ M (micromolar) respectively. MEHP at 50 to 100 μ M (micromolar) did not inhibit FSH induced cumulus expansion of bovine oocytes. In 2009, Lenie et al. [12] demonstrated that 50 μ M was the minimum concentration of MEHP required to cause an abnormal increase in the ratio of testosterone to estradiol, and the overall levels of progesterone produced. Administration of DEHP to porcine cumulus-oocyte complexes in vitro, at levels ranging from 100 pM (picomolar) to 100 μ M, had no effect on the cumulus expansion or meiotic maturation of porcine oocytes.

This is the third study to determine if bisphenol A preferentially accumulates in the ovarian follicular microenvironment. In the current study, no bisphenol A was detected in the follicular fluid of any of the five patients which differs from two previous studies [25, 30]. In 2002, Ikezuki et al. [30] measured bisphenol A in non-pregnant Japanese patients yielding serum levels which averaged 2.0 ng/mL

Table 1 Follicular fluid levels of bisphenol A and phthalates

Specimen #	1	2	3	4	5	Mean +/- Standard Deviation
Total specimen Volume (mL)	66.5	60.5	60	109.5	90.5	77.4 +/- 21.8
Media Volume (mL)	23	17.5	9.5	29.5	68.5	29.6 +/- 23.0
Follicular Fluid Volume (mL)	43.5	43	50.5	80	22	47.8 +/- 20.9
Bisphenol A (BPA)	U	U	U	U	U	U
Monomethyl phthalate (ng/mL) (m-MP)	1.37	U	U	1.01	U	1.19 +/- 0.25
Monoethyl phthalate (ng/mL) (m-EP)	2.10	1.49	1.20	8.39	2.78	3.19 +/- 2.97
Mono-n-butyl phthalate (ng/mL) (m-BuP)	1.48	1.45	1.08	1.46	2.63	1.62 +/- 0.59
Monobenzyl phthalate (ng/mL) (m-BzP)	U	U	U	U	U	U
Mono-2-ethylhexyl phthalate (ng/mL) (m-EHP)	11.4	5.96	7.62	14.1	7.61	9.34 +/- 3.33
Mono-(2-ethyl-5-oxohexyl) phthalate (DEHP Metabolite VI) (ng/mL) (m-EOHP)	U	U	U	U	U	U
Mono-(2-ethyl-5-hydroxyhexyl) phthalate (DEHP Metabolite IX) (ng/mL) (m-EHHP)	U	U	U	1.43	U	1.43

U Undetectable

($n=37$) and follicular fluid levels averaged 2.4 ng/mL ($n=32$). In the 2005 Tsutsumi study [25], serum and follicular fluid levels ranged 1–2 ng/mL. Conversion of standard units demonstrates that one ng/mL of Bisphenol A corresponds with a 4.4 nanomolar (nM) concentration.

The levels of bisphenol A in human follicular fluid, just as in phthalates, are several orders of magnitude below minimum levels known to cause reproductive toxicity to the oocytes of laboratory animals. In 2008, Eichenlaub-Ritter et al. [10] tested the effects of varying concentrations of bisphenol A, ranging from 50 ng/mL to 10 μ g/mL, on the meiotic spindle of murine oocytes, and found that 3.75 μ g/mL was the minimum level to adversely affect the spindle and chromosomal segregation. According to a study in 2005 [7] only Bisphenol A levels greater than 100 μ M resulted in disruption of the Ca^{++} oscillations required in murine oocytes for polar body extrusion. In 2008, Lenie et al. [11] demonstrated that a 30 μ M dose was the minimum concentration of Bisphenol A to negatively impact cumulus cell expansion and estrogen production by murine granulosa cells in vitro. These findings were corroborated in a study [13] which showed that 100 picomolar to 1 μ M concentrations of Bisphenol A had no effect on FSH-induced cumulus expansion of porcine cumulus-oocyte complexes. Only at a concentration of 100 μ M did Bisphenol A significantly decrease progesterone production by porcine cumulus-oocyte complexes. The difference in detectable levels of bisphenol A in our patient population compared to others suggests that levels may depend on population density or study location. Household and medical products used in Japan may contain higher levels of bisphenol A than those used in the Northeastern United States.

A criticism of this study could be the small sample size, however, the results demonstrate that accumulation of both phthalates and bisphenol A in human follicular fluid in vivo are minimal. The levels phthalates and bisphenol A in human follicular fluid are 200–1000 fold lower than the levels required to produce detrimental effects on oocyte meiotic maturation, cumulus expansion or hormone production in laboratory animals in vitro. Therefore, phthalates and bisphenol A in the human ovarian microenvironment of an unselected population are not clinically significant as ovulatory disruptors or reproductive toxins, even in the Northeastern United States where there is a high level of industrial activity. Further investigation of phthalate and bisphenol A accumulation in vivo in human follicular fluid may not be productive.

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