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ASSISTED REPRODUCTION TECHNOLOGIES

Associations between toxic metals in follicular fluid and in vitro fertilization (IVF) outcomes

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Abstract
Purpose We previously reported associations between trace concentrations of Hg, Cd and Pb in blood and urine and reproductive outcomes for women undergoing in-vitro fertilization (IVF). Here we assess measurements in single follicular fluid (FF) specimens from 46 women as a presumably more relevant marker of dose for reproductive toxicity.
Methods FF specimens were analyzed for Hg, Cd and Pb using sector field-inductively coupled plasma-mass spectrometry (SF-ICP-MS). Variability sources were assessed by nested ANOVA. Multivariable regression was used to evaluate associations for square root transformed metals with IVF outcomes, adjusting for confounders.
Results An inverse association is detected for FF Pb and fertilization (relative risk (RR)=0.68, P=0.026), although positive for Cd (RR=9.05, P=0.025). While no other statistically significant associations are detected, odds ratios (OR) are increased for embryo cleavage with Hg (OR=3.83, P=0.264) and Cd (OR=3.18, P=0.644), and for embryo fragmentation with Cd (OR=4.08, P=0.586) and Pb (OR=2.22, P=0.220). Positive estimates are observed for Cd with biochemical (RR=19.02, P=0.286) and clinical pregnancies (RR=38.80, P=0.212), yet with very low precision.
Conclusions We have identified associations between trace amounts of Pb and Cd in FF from a single follicle, and oocyte fertilization. Yet, the likelihood of biological variation in trace element concentrations within and between follicles, coupled with levels that are near the limits of detection suggest that future work should examine multiple follicles using a 'one follicle-one oocyte/embryo' approach. A larger study is merited to assess more definitively the role that these environmental factors could play with respect to egg quality in IVF programs.

Keywords Cadmium (Cd) · Follicular fluid (FF) · in vitro fertilization (IVF) · Lead (Pb) · Mercury (Hg) · Reproductive outcomes

Capsule Substantial within-woman biologic variability for the concentrations of metals measured in follicular fluid specimens necessitates a 'one follicle-one oocyte/embryo' approach to studies of background exposures and IVF outcomes.

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Introduction

Human female reproductive toxicity, in terms of reduced fecundity and fertility, has been reported in association with high dose and occupational level exposures to mercury (Hg), cadmium (Cd), and lead (Pb) [1–3]. Associations are reported at high doses among couples conceiving with assisted reproductive technologies as well as those conceiving unassisted [4, 5]. Given their widespread distribution [6], persistence in vivo, and potential for reproductive

toxicity at trace concentrations, Hg, Cd and Pb are of concern [1–3]. Yet, the results from studies assessing reproductive toxicity associated with trace exposures to Hg, Cd and Pb in women have been inconsistent. Studies report decreased fecundity or fertility [7–12], no association [13, 14], or even increased fecundity [7, 15] in association with higher exposures. In our studies, we conducted preliminary evaluations of trace metal concentrations measured in blood and urine specimens collected from women undergoing in vitro fertilization (IVF), and reported associations between blood Pb and oocyte maturity [16, 17], urine Cd and oocyte fertilization [17], blood Pb level and embryo cleavage [18], and blood Hg and Cd and embryo implantation [19].

Follicular fluid (FF) is comprised of a blood plasma ultrafiltrate, with selective exclusion of high weight molecular proteins facilitated by the blood-follicle-barrier, that fills the growing follicle and bathes a developing oocyte [20]. The constituents of FF may reflect environmental exposures relevant to the early stages of human reproduction including the quality of a developing oocyte and embryo. It is presumed that FF will provide a closer approximation to the biologically-effective dose of a toxicant to an oocyte than achieved using blood or urine measures. Several groups previously reported Hg, Cd and/or Pb concentrations measured in human FF specimens, although with little methodologic detail [7, 12, 15, 21–23, 24]. To facilitate investigation of associations between toxic metals in FF and IVF outcomes, our laboratory recently developed and validated a method for the measurement of elements in FF at trace concentrations [25]. Here, we augment our previous dataset including blood and urine concentrations of toxic trace metals [17] with concentrations of Hg, Cd and Pb measured in single FF specimens collected from women undergoing IVF at our northern California clinic.

The aim of this preliminary study is to assess the relative importance of Hg, Cd and Pb measured in a single FF specimen in assessing reproductive toxicity during IVF, compared to our prior work using blood and urine measures.

Methods

Sample selection and clinical protocol

Fifty-eight female patients undergoing IVF treatment at the University of California at San Francisco (UCSF) Center for Reproductive Health were recruited to the Study of Metals and Assisted Reproductive Technologies (SMART) between March 12th, 2007 and April 29th, 2008. Sample selection and clinical protocols were previously described in detail [17, 18]. Briefly, female patients underwent gonadotropin-induced ovarian stimulation per clinic protocols. When two or more follicles exceeded ≥17 mm diameter, human chorionic gonadotropin (hCG) was administered and oocytes were retrieved 36 h later. Collected oocytes in metaphase-II (MII) arrest were fertilized by conventional insemination or by intracytoplasmic sperm injection (ICSI) using sperm from male partners obtained on the day of oocyte retrieval or from a donor. Approximately 16–18 h following insemination, zygotes were identified by the appearance of two pronuclei. A single embryologist who was blinded to exposure data examined all embryos produced by a couple as previously described in detail [18]. Embryo cell number (ECN) was assessed on the day of transfer and characterizes cleavage or growth rate; it is a positive predictor for IVF success. Embryo fragmentation score (EFS) was assessed approximately 48 h after fertilization and is an inverse predictor for IVF success [26]. Embryos were transferred on the second or third day post-fertilization. Pregnancy outcome was assessed using a quantitative serum beta hCG ELISA test conducted 14 days following embryo transfer, followed by a second test confirming 'biochemical pregnancy' 2–3 days later if positive. 'Clinical pregnancy' was confirmed by ultrasound visualization of one or more gestational sacs 2 weeks later [27].

Follicular fluid specimens were collected into empty syringes during oocyte retrieval, using a transvaginal ultrasound probe with an 18 gauge, 36 cm oocyte aspiration needle mounted on a needle guide directly attached to the probe. Between 0.5 mL and 5.0 mL FF was aspirated from a single large follicle in each ovary, spun for 10 min at 1500 × g, aliquoted into empty 1.8 mL cryovials until exhausted and then stored at −80 °C [25]. Follicular fluid specimens were collected from 48 women, including 40 women with specimens from a single follicle and eight women with specimens from two follicles. Blood and urine specimens were also collected at the time of oocyte retrieval and have previously been described in detail for the overall study cohort [17, 28]. Informed consent was obtained from all study participants prior to inclusion in the study. The study protocol was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki as approved by the UCSF Committee for Human Research, and the Institutional Review Boards at the University at Albany and the New York State Department of Health.

Exposure assessment

During the spring of 2011, FF specimens were analyzed for Hg, Cd, and Pb using a laboratory developed method that we optimized and validated for a Thermo Fisher Scientific Element2 sector field-inductively coupled plasma-mass spectrometer (SF-ICP-MS) (Bremen, Germany) [25]. Serum reference materials were used to assess accuracy and precision for FF due to lack of any commercially available FF reference materials. We used serum quality controls and

spiked pooled FF to validate the optimized method. The limits of detection (LODs) were defined as three times the standard deviation of the quantity measured in matrix blanks and are equal to 0.2 μg/L for Hg, 0.02 μg/L for Cd, and 0.03 μg/L for Pb. All laboratory values were reported irrespective of the LODs; this distribution-free approach precludes bias that may be introduced into regression models by censoring values below LODs [29]. Specimens were carefully examined for potential blood contamination; two pink specimens were excluded. A total of 46 women were thus included in this analysis.

Blood specimens were previously analyzed for Hg, Cd, and Pb, and urine specimens were previously analyzed for Cd and additional trace elements using a method optimized for a Perkin Elmer Sciex ELAN DRC II ICP-MS (PerkinElmer Life and Analytical Sciences, Shelton, CT) with dynamic reaction cell (DRC) technology [17, 28]. The analytic procedures for blood [30] and urine [31] specimens have been previously described in detail. The LODs were 0.2 μg/L for blood Hg, 0.2 μg/L for blood Cd, 0.17 μg/dL for blood Pb, and 0.02 μg/L for urine Cd.

Statistical analysis

Distributions were characterized for metal concentrations and relevant demographic and clinical factors. Spearman rank correlation coefficients and the Mann–Whitney *U*-test were used to assess bivariate associations among metal concentrations in FF, blood, and urine. The proportion of mature oocytes was defined as the total number of oocytes in MII-arrest divided by the total number of oocytes collected from women undergoing ICSI. The proportion of oocytes fertilized comprised the total number of zygotes formed divided by the total number of mature oocytes injected from women undergoing ICSI, or divided by the total number of oocytes with a visible polar body observed at fertilization for women undergoing conventional insemination. Following normalization by log transformation with the addition of a constant to accommodate negative values (i.e., 1.00), we characterized sources of measurement variability using nested ANOVA for seven women with FF specimens from two different follicles [32].

Modified Poisson regression was used to evaluate associations between IVF endpoints including oocyte maturity (MII-arrest among ICSI cases, no/yes), oocyte fertilization (zygote, no/yes), biochemical pregnancy (positive hCG tests, no/yes) and clinical pregnancy (gestational sac on ultrasound, no/yes), and the concentrations of FF Hg, Cd, and Pb [33, 34]. All multivariable models were adjusted for age, cigarette smoking, and race/ethnicity as confounding variables identified using the literature in conjunction with directed acyclic graphs (DAGs) [35]. Employing causal graphing theory, DAGs articulate minimum covariate sets to control confounding under a postulated causal framework. Prior to multivariable analysis, a constant was added to accommodate negative values (i.e., 0.01) and FF metal concentrations were square root transformed to stabilize variances. Generalized estimating equations (GEE) were used to provide robust standard errors [36]. Single metal models for each Hg, Cd or Pb as the sole predictor of interest, and multiple metal models including Hg, Cd and Pb simultaneously as predictors of interest, were generated for each IVF endpoint. Exponentiation of model coefficients and their 95 % confidence intervals (CIs) provide relative risks (RR) and 95 % CIs for a single square root-transformed μg/L (√μg/L) increase in FF metal concentrations.

Multivariable ordinal logistic regression models were employed to estimate associations between FF metal concentrations and embryo quality outcomes. Collected embryos were grouped into tertiles of ECN (1–5 cells/6–7 cells/8–12 cells) and EFS (1/2/3-5) [18]. Under the assumption of proportional odds, ordinal logistic regression estimates the log-odds of a subject's outcome falling into a higher ordered endpoint category [37]. Odds ratios (OR) and 95 % CIs for a single √μg/L increase in FF metal concentrations, adjusted for age, cigarette smoking and race/ethnicity, and limited to day 3 embryo transfer for ECN, were estimated by exponentiation of ordinal logistic regression model coefficients and their CIs. Statistical significance was defined as $P<0.05$ for a two-tailed test. SAS v.9.2 (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Demographic, clinical and exposure factors

The distribution of demographic and clinical variables for 46 women providing FF specimens is described in Table 1. Approximately 30 % of participants are Asian, with a majority reporting a non-Asian race/ethnicity, primarily non-Hispanic white. Seventy-two percent of women had ICSI, which is evidenced by the high proportion of "unexplained" (34.8 %) and "male factor" (26.1 %) diagnoses. Between 2 to 26 oocytes were collected from each study participant. On average, 0.82 oocytes collected from ICSI cases were in MII-arrest and 0.65 oocytes were normally fertilized for both ICSI and conventional insemination cases, with between 1 and 14 embryos produced by each woman. Most embryos were transferred on day 3 (79.5 %).

Distributions of metals are presented in Table 2 for women with FF specimens. Median (range) FF Hg and Pb concentrations are 0.65 μg/L (<0.2–1.68) and 0.17 μg/L (<0.03–1.93), respectively, and over 90 % of measured values are above the LODs. Median (range) FF Cd is 0.02 μg/L (<0.02–0.10), with 54.3 % of values above the LOD. Likewise, 70 % of values are above the LOD for blood Cd, while all blood Hg and Pb values are above the

Table 1 Distribution of demographic and clinical variables for in vitro fertilization (IVF) patients with follicular fluid metals; the Study of Metals and Assisted Reproductive Technology (SMART)

Variables	n	Mean (%)	SD	Min.	25th %tile	Median	75th %tile	Max.
Patients:								
Age (years)	46	35.6	3.7	28	33	36	38	43
Body mass index (kg/m^2)	44	23.6	3.4	17.9	21.1	23.0	25.2	32.0
Ever cigarette smoker [a]	7	(15.2)	-	-	-	-	-	-
Asian race/ethnicity	14	(30.4)	-	-	-	-	-	-
Total oocytes collected	46	12.3	6.6	2.0	7.0	11.0	16.0	26.0
Total embryos produced	44	6.8	3.6	1.0	4.5	6.0	10.0	14.0
Intracytoplasmic sperm injection	33	(71.7)	-	-	-	-	-	-
Primary infertility diagnosis:								
Unexplained	16	(34.8)	-	-	-	-	-	-
Male factor	12	(26.1)	-	-	-	-	-	-
Diminished ovarian reserve	9	(19.6)	-	-	-	-	-	-
Tubal factor	4	(8.7)	-	-	-	-	-	-
Other [b]	5	(10.9)	-	-	-	-	-	-
IVF endpoints:								
Proportion mature oocytes [c]	46	0.82	0.19	0.33	0.67	0.85	1.00	1.00
Proportion fertilized oocytes [d]	46	0.65	0.27	0.00	0.55	0.67	0.86	1.00
Mean embryo cell number	44	5.6	1.7	2.0	4.7	5.7	7.1	8.4
Mean embryo fragmentation score	43	2.2	0.8	1.0	1.7	2.0	2.7	4.0
Day 3 embryo transfer	35	(79.5)	-	-	-	-	-	-
Biochemical pregnancy [e]	22	(52.4)	-	-	-	-	-	-
Clinical pregnancy [f]	18	(42.9)	-	-	-	-	-	-

[a] Self-reported active or past cigarette smoking; [b] includes 2 cases of endometriosis and 3 cases of structural defects; [c] defined as the average proportion of oocytes recovered in metaphase-II (M2) arrest; [d] defined as the average proportion of fertilized oocytes with the presence of two pronuclei and two Barr-bodies; [e] defined as two positive serum human chorionic gonadotropin (hCG) tests; [f] defined as visualization of at least one gestational sac by ultrasound

Max maximum value, *Min* minimum value, *SD* standard deviation

LOD, and all urine Cd values are above the LOD. Follicular fluid metal concentrations are considerably lower on average than those measured in blood and urine. A majority of the variability in FF Hg measures is attributed to factors between women (62.2 %); small components are contributed by factors within woman (15.8 %) and by analytic factors (22.0 %). In contrast, the variability in FF Cd is due overwhelmingly to analytic factors (i.e., most values are close to the LOD), with negligible variability between or within woman. For FF Pb, a majority of the variability is attributed to factors within woman (58.0 %), yet a substantial contribution is made by analytic factors (42.0 %).

Correlations among metal concentrations in FF, blood, and urine are presented in Table 3. Statistically significant correlations are detected for FF Hg and blood Hg (r=0.64, P<0.0001), FF Cd and blood Cd (r=0.59, P<0.0001), and blood Cd and urine Cd (r=0.51, P=0.001). Median FF Hg is higher (P=0.023) for Asian women (0.92 μg/L, range 0.69–1.30) than for non-Asian women (0.79 μg/L, range 0.45–1.16). Median FF Pb concentration varies by diagnosis (P=0.066), although in a 'borderline' significant fashion, in which women with male partner infertility demonstrate the highest median value (0.47 μg/L, range 0.20–0.84), followed closely by women with unexplained infertility (0.45 μg/L, range 0.27–0.86), and women with diminished ovarian reserve have the lowest median value (0.27 μg/L, range 0.16–0.52). The proportion of normally fertilized oocytes is positively correlated to FF Hg (r=0.30, P=0.045) and FF Cd (r=0.28, P=0.062), and inversely to FF Pb (r=−0.26, P=0.081); the latter are of 'borderline' significance.

Multivariable analysis

The results of the modified Poisson regression analysis for oocyte maturity and fertilization are presented in Table 4. No associations are indicated between FF metals and oocyte maturity, although point estimates are positive for FF Hg and FF Cd in regression models including a single predictor metal and confounders. A similar pattern of non-significant

Table 2 Distribution of metals measured in follicular fluid (FF), blood, and urine from in vitro fertilization (IVF) patients with FF metals; the Study of Metals and Assisted Reproductive Technology (SMART)

Metals	n	Mean	SD	Min.	25th %	Median	75th %	Max.	% above the LOD
Follicular fluid (μg/L):									
Mercury	46	0.69	0.30	< 0.2 [a]	0.53	0.65	0.82	1.68	97.8
Cadmium	46	0.03	0.02	< 0.02 [a]	< 0.02 [a]	0.02	0.04	0.10	54.3
Lead	46	0.25	0.31	< 0.03 [a]	0.10	0.17	0.26	1.93	93.5
Blood (μg/L):									
Mercury	40	3.03	1.96	0.28	1.60	2.96	3.87	8.77	100.0
Cadmium	40	0.35	0.22	< 0.2 [a]	< 0.2 [a]	0.27	0.44	0.96	70.0
Lead (μg/dL)	21	0.82	0.31	0.34	0.62	0.85	1.05	1.28	100.0
Urine (μg/g creatinine):									
Cadmium	45	0.31	0.18	0.04	0.18	0.31	0.40	0.98	100.0

[a] Value less than the limit of detection (LOD)

Max maximum value, *Min* minimum value, *SD* standard deviation

Blood and urine values comprise a subset of the values reported previously for the full SMART cohort [17]

positive associations between FF Hg and FF Cd and oocyte maturity, with effect estimates close to 1.0 for FF Pb is apparent when simultaneously including three metals as predictors. We detect a statistically significant positive association between FF Cd and oocyte fertilization (RR=8.94, 95%CI 1.32–60.49) when considered in isolation and adjusted for confounders, and again when considered in the context of FF Hg and FF Pb (RR=9.05, 95%CI 1.32–61.81). In contrast, we detect a statistically significant and inverse association for FF Pb concentration and oocyte fertilization in the context of FF Hg, FF Cd and confounders (RR=0.68, 95%CI 0.49–0.96). The point estimate for FF Pb as an isolated predictor of fertilization is also negative.

Table 5 presents the ordinal logistic regression models describing associations between FF metals and embryo quality indicators, again considering each metal as the sole predictor of interest (i.e., single metal models) and then as a group member (i.e., multiple metals models). No statistically significant associations are detected. Yet, in the multiple metals model the point estimates for ECN with FF Hg (RR=3.83, 95%CI 0.36–40.51) and Cd (RR=3.18, 95%CI 0.02–430.78) are positive, albeit with wide confidence intervals; the pattern is similar for the single metal models. Point estimates for the association between EFS and FF Cd (RR=4.08, 95%CI 0.03–639.70) and FF Pb (RR=2.22, 95%CI 0.62–7.89) are also positive in the multiple metals model, with wide confidence intervals, and with a similar non-significant pattern observed for single metal models.

The results of the modified Poisson regression analyses for biochemical pregnancy and clinical pregnancy as a function of FF metals, considered singly (i.e., single metal models) and considered as a panel (i.e., multiple metals models)

Table 3 Correlations (P-value) among metal concentrations in follicular fluid (FF), blood, and urine in 46 *vitro* fertilization (IVF) patients; the Study of Metals and Assisted Reproductive Technology (SMART)

	FF Hg	FF Cd	FF Pb	Blood Hg	Blood Cd	Blood Pb	Urine Cd [a]
FF Hg	—	−0.10	0.03	**0.64**	0.04	−0.21	0.28
		(0.505)	(0.853)	**(<0.0001)**	(0.797)	(0.360)	(0.181)
FF Cd		—	0.11	0.13	**0.59**	−0.12	0.28
			(0.453)	(0.440)	**(<0.0001)**	(0.618)	(0.07)
FF Pb			—	−0.02	0.13	0.19	0.10
				(0.914)	(0.419)	(0.401)	(0.515)
Blood Hg				—	0.28	−0.06	0.28
					(0.082)	(0.810)	(0.092)
Blood Cd					—	−0.013	**0.51**
						(0.955)	**(0.001)**
Blood Pb						—	−0.05
							(0.854)

[a] Adjusted for urine creatinine concentration (mg/dL)

P<0.05 in bold type

Table 4 Modified Poisson regression models of in vitro fertilization (IVF) outcomes on follicular fluid metals (square root transformed) measured in patients, with generalized estimating equations used to generate robust standard errors; the Study of Metals and Assisted Reproductive Technology (SMART)

Metals (√μg/L)	n women/ n oocytes	Single metal model:			Multiple metals model:		
		RR [a]	95 % CI		RR [b]	95 % CI	
Oocyte maturity: [c]							
Mercury	33/396	1.32	0.85	2.04	1.27	0.80	2.02
Cadmium	33/396	1.79	0.59	5.43	1.63	0.45	5.94
Lead	33/396	0.90	0.57	1.43	0.95	0.59	1.51
Oocyte fertilization: [d]							
Mercury	46/475	2.08	0.94	4.61	1.98	0.84	4.67
Cadmium	46/475	8.94 *	1.32	60.49	9.05 *	1.32	61.81
Lead	46/475	0.76	0.52	1.10	0.68 *	0.49	0.96
Biochemical pregnancy: [e]							
Mercury	42	0.81	0.14	4.55	0.75	0.11	4.97
Cadmium	42	21.78	0.09	5422.03	19.02	0.09	4247.57
Lead	42	1.76	0.44	7.05	1.64	0.47	5.69
Clinical pregnancy: [f]							
Mercury	42	0.50	0.05	5.18	0.44	0.03	6.25
Cadmium	42	39.27	0.12	13,291.60	38.80	0.12	12,186.31
Lead	42	1.64	0.31	8.79	1.55	0.35	6.87

[a] Relative risk (RR) as a function of concentrations of a single measured FF metal adjusted for age, cigarette smoking and race/ethnicity; [b] RR as a function of concentrations of all measured metals adjusted for age, cigarette smoking and race/ethnicity; [c] cases of intracytoplasmic sperm injection (ICSI) only, defined as oocyte recovered in metaphase-II (M2) arrest; [d] defined as presence of two pro-nuclei and two Barr-bodies; [e] defined as two positive serum human chorionic gonadotropin (hCG) tests; [f] defined as visualization of one or more gestational sacs on ultrasound; * $P<0.05$

CI confidence interval exposure

are described in Table 4. No effect estimates approach statistical significance, yet a large and unexpected positive point estimate is observed for Cd when assessed as the sole predictor of interest (RR=21.78, 95%CI 0.09–5422.03) or in the context of FF Hg and FF Pb (RR=19.02, 95%CI 0.09–4247.57). However, the confidence intervals are very wide. Effect estimates for FF Hg are less than one in those models, although not statistically significant. A similar

Table 5 Ordinal logistic regression models of embryo quality on concentrations of follicular fluid metals (square root transformed) measured in female in vitro fertilization (IVF) patients, with generalized estimating equations used to generate robust standard errors; the Study of Metals and Assisted Reproductive Technology (SMART)

Metals (√μg/L)	n (women)/ n (embryos)	Single metal models:			Multiple metals models:		
		OR [a]	95 % CI		OR [b]	95 % CI	
ECN: [c]							
Mercury	34/273	4.37	0.58	32.71	3.83	0.36	40.51
Cadmium	34/273	6.11	0.07	574.44	3.18	0.02	430.78
Lead	34/273	0.92	0.23	3.72	0.84	0.21	3.42
EFS:							
Mercury	43/285	1.59	0.19	40.78	1.24	0.15	10.41
Cadmium	43/285	7.66	0.06	992.67	4.08	0.03	639.70
Lead	43/285	2.38	0.63	9.03	2.22	0.62	7.89

[a] Odds ratio (OR) for an embryo as a function of concentrations of a single measured metal adjusted for age, cigarette smoking and race/ethnicity; [b] odds ratio for an embryo as a function of concentrations of all measured metals adjusted for age, cigarette smoking and race/ethnicity; [c] day three embryo transfers only

CI confidence interval

pattern is observed when clinical pregnancy is the outcome of interest, with more extreme and positive effect estimates for Cd considered alone (RR=39.27, 95%CI 0.12–13,291.60), or when considered in conjunction with FF Hg and FF Pb (RR=38.80, 95%CI 0.12–12,186.31). Again, FF Hg effect estimates are less than one in both models and not statistically significant.

Discussion

The results of this study indicate an inverse association for FF Pb and oocyte fertilization, as well as a somewhat counterintuitive positive association between FF Cd and oocyte fertilization among women undergoing IVF. We also report unexpected strong and positive point estimates for FF Cd and pregnancy, coupled to negative estimates for FF Hg, although the confidence intervals are wide. Yet, FF Cd concentrations occur close to the LOD, introducing increased relative uncertainty into the analysis. Results for embryo quality indicators suggest increased cleavage rate as well as increased embryo fragmentation in association with various metal exposures, yet the results are imprecise. These data expand upon our prior studies of Hg, Cd and Pb measured in blood and urine specimens collected from the same study population, in an effort to assess the relative importance of FF metal concentrations which is presumably a more pertinent biomarker of oocyte exposure in the ovarian follicle. However, our results suggest that variability between women makes only a minor contribution to the overall variability in FF metal concentrations, and there is limited consistency with our prior work. Thus assessment of trace metals in a single FF specimen may not in fact be an improvement over blood and/or urine.

Associations among metals in FF, blood and urine compartments

The FF concentrations of Hg, Cd and Pb measured in our study are generally lower than reported in the literature [7, 12, 16, 21–23], a comprehensive comparison of which is provided elsewhere [25]. The lower FF values measured in our study may reflect the highly sensitive ICP-MS method employed, coupled to different exposure patterns. Further studies will be necessary to establish reference ranges to facilitate interpretation of biomonitoring data. Higher FF Hg concentrations among Asian women are detected, consistent with prior reports for blood Hg in this study population [17] and others [38]. We also detect moderate positive correlations between concentrations of FF Hg and blood Hg, and concentrations of FF Cd and blood Cd, although no correlation for FF Pb and blood Pb. Follicular fluid is a type of blood plasma ultra-filtrate, sharing a similar yet modified chemical composition [20] and we thus anticipated positive correlations between these two compartments for all metals as reported previously [23]. However, the large component of variance attributed to analytic factors and the large relative uncertainty in values that are close to the LOD likely overshadowed any association.

Associations between metals and oocyte maturity

No associations are suggested between FF Hg, Cd or Pb and oocyte maturity among IVF patients in the current study. We previously suggested a four-fold increase in the probability for retrieval of an immature oocyte in association with each 1 μg/dL increase in blood Pb in this study population, albeit with a wide confidence interval [16, 17]. The discrepancy in this study may indicate the absence of an underlying association between Pb and oocyte maturity at the trace-level exposures experienced by our study population. However, the discrepancy might also reflect non-differential exposure measurement misclassification secondary to variation in sampled follicle diameter and other sources of variability within woman, as well as analytic factors. The degree to which follicle analyte levels reflect blood levels appears to vary by analyte, and FF concentrations increasingly reflect blood concentration with increasing diameter [23]. We aspirated single follicles over 17 mm, however actual diameter was not recorded, precluding adjustment, and single oocytes/embryos were not tracked. Given the fairly low Pb concentrations measured in FF in our study sample, it is difficult to elucidate the degree of bias resulting from such exposure misclassification, although we suspect it to be non-differential and towards the null hypothesis. To our knowledge, no studies have previously addressed associations between oocyte maturity and FF concentrations of Hg, Cd or Pb. A future study, employing a ‘one follicle-one oocyte’ design and adjustment for follicle diameter to potentially accommodate chemical ‘dilution’ will be necessary to address this issue further.

Associations between metals and oocyte fertilization

In contrast to models for oocyte maturity, we detect a statistically significant 32 % decrease in the probability for oocyte fertilization per 1 √μg/L increase in FF Pb, as well as a nine-fold increase per 1 √μg/L increase in FF Cd. Our prior research suggests an increase in oocyte fertilization of 41 %–48 % in association with female urine Cd concentrations, although not of statistical significance [17, 28], and not for Cd in blood [28]. A prior investigation of 619 Saudi Arabian IVF patients reported a positive yet nonsignificant association between FF Pb and oocyte fertilization (OR=1.45, 95%CI 0.69–3.02), with a ‘borderline-significant’ positive association for FF Cd (OR=1.87, 95%CI 0.91–3.82) in

the same logistic regression model including 19 covariates [7]. An earlier, and smaller study reported significantly higher FF Cd concentrations for three Canadian IVF patients who achieved pregnancy, compared to three IVF patients with no fertilized oocytes [15].

Our FF Cd results are consistent with prior publications reporting a positive association with fertilization, yet our FF Pb results are inconsistent with the single prior study reporting an association. The latter observation is consistent with experimental data suggesting that Pb^{2+} may interfere with cytosolic Ca^{2+} initiated resumption of meiosis following gamete fusion [39]. In addition, direct oxidation of membrane lipids, and depletion of anti-oxidants due to a strong affinity for the thiols plentiful in glutathione [40], may account for the inhibitory effect of Pb; a narrow range of reactive oxygen species is critical to proper oocyte development and fertilization [41]. Cadmium too elicits increases in reactive oxygen species [40], as well as alteration of cytosolic Ca^{2+} levels, yet most animal studies report reduced oocyte fertilization [42]. It is possible that the association we observe for FF Cd is confounded by associations with one or more essential elements associated with oocyte fertilization, such as FF Zn [43]. Yet, we do not detect a correlation between FF Cd and FF Zn in our data set (data not shown). The sparse nature of this literature, complex analytical challenges, and the limited number of participants in our study preclude any conclusions and underscore the need for additional investigation.

Associations between metals and embryo quality

We previously reported an inverse association between blood Pb concentrations in IVF patients and embryo cleavage rate measured as ECN (OR=0.25, 95%CI 0.07–0.86), adjusted for age, cigarette smoking, race/ethnicity, blood Hg and urine Cd concentrations; we detected no association for EFS [18]. We do not detect an association in this study for Hg, Cd or Pb with ECN or with EFS. Yet, we observe positive point estimates of moderate magnitude between FF Hg, FF Cd and ECN, adjusted for FF Pb, age, race/ethnicity and cigarette smoking, although, not of statistical significance. Positive point estimates suggesting increased embryo fragmentation are also observed for all metals with respect to EFS, with moderate effects for FF Hg and Cd, yet confidence intervals are wide. We are unable to identify previous human studies describing associations between FF metals and embryo quality indicators per se. However, the aforementioned Saudi Arabian IVF study reports an inverse association between blood Cd and the number of 'poor embryos' (r=−0.09, P=0.04), yet no association was found for FF Hg, Cd or Pb [7]. In addition, the earlier Canadian IVF study reported no association between measured FF Cd and embryo quality [15]. Embryotoxic effects have been reported following embryo treatment in vitro with low levels of Cd and Pb in the experimental literature [44, 45]. Inconsistencies between our prior and current results may again indicate the absence of an association at the study population level, yet the very low and limited range of metal concentrations measured in FF specimens are likely to compromise study power, necessitating a substantial number of participants to detect an association. This may be further obscured by the previously described issues related to FF exposure misclassification. The sparse literature concerning this topic hinders interpretation of our results. A large scale study is clearly necessary to elucidate the role played by FF metals and embryo quality during IVF.

Associations between metals and pregnancy

Though not of statistical significance, associations between FF metals and pregnancy in the current study also conflict with our previous work involving blood and urine specimens. The point estimates for FF Cd with biochemical pregnancy and clinical pregnancy adjusted for FF Hg, FF Pb and confounders are positive and large, yet the confidence intervals are very wide and include the null hypothesis (i.e., 1.0). In contrast, we recently reported an inverse association for blood Cd concentrations [19], adjusted for blood Hg and Pb, age, race/ethnicity, and cigarette smoking among women from the same study population (RR=0.18, 95%CI 0.03-0.94). Yet, the negative point estimates for FF Hg with biochemical and clinical pregnancy in the same multivariable models as FF Cd are consistent with the inverse, yet non-significant point estimates for blood Hg adjusted for blood Cd and Pb, and confounders in our prior study (RR=0.79, 95%CI 0.60–1.06 and RR=0.80, 95%CI 0.58–1.11, respectively). Furthermore, our prior work reports a significant inverse association for blood Hg and biochemical (RR=0.65, 95%CI 0.44–0.96) and clinical pregnancies (RR=0.67, 95%CI 0.48–0.92), adjusted for urine Cd and creatinine, blood Pb, age, race/ethnicity, and cigarette smoking.

Given the discrepancies between the results of the current study and our prior study of pregnancy in this population, it is tempting to speculate that the target tissues for Cd- or Hg-associated toxicity during IVF may be at the level of implantation or maintenance of early pregnancy rather than from direct embryotoxicity, and thus FF metals may be a less relevant indicator of biologically effective dose than are blood or urine concentrations. With respect to implantation and clinical pregnancy the exposures to sperm may in fact be of equal or greater relevance than exposures to the oocyte [46]. No association was suggested for FF Hg, Cd or Pb concentrations and biochemical pregnancy during multivariable regression in the aforementioned Saudi Arabian IVF study [7]; in fact the unadjusted association suggested a

'borderline significant' increase in pregnancy for each 1 μg/L increase in FF Hg (OR=1.17, 95%CI 0.98–1.40). However, a cross-sectional study of women attending an infertility or a postpartum clinic reported a higher odds for infertility among women with blood Pb exceeding 2.5 μg/dL [9]. Furthermore, a recent prospective study of 501 couples conceiving unassisted [8] reported a 22 % reduced probability for conception per 1 μg/L increase in blood Cd adjusted for blood Hg, blood Pb and covariates (OR=0.78 95%CI 0.63–0.97), yet no association for Hg or Pb. Yet, an earlier study of women undergoing IVF in Rhode Island (U.S.) reported significantly lower FF Pb concentrations among four women whose procedure resulted in pregnancy compared to five women whose procedure did not [12]. Additional work is clearly needed to assess more definitively the impact of FF trace metals on pregnancy.

Limitations and strengths of the study

The current study presents with important limitations and the results should thus be interpreted with caution. Foremost of these is the limited sample size, which restricts our ability to detect associations and results in imprecise estimates for the effect of FF metals. We are also limited by an inability to stratify by factors likely to modify the association between FF metals and IVF outcomes, including diagnosis, type of stimulation and IVF protocol, as well as demographic factors such as race/ethnicity. Our bivariate analysis results suggest that blood Pb concentration varies with infertility diagnosis, yet we are unable to assess the importance more thoroughly. Unrecognized 'effect modification' by such factors may bias the reported results. Furthermore, our results are vulnerable to confounding by unmeasured factors causally related to both exposure and outcome, such as related environmental exposures, health behaviors, or diet factors that may be of importance. This scenario might account for unanticipated results, for example if such a factor increases the risk for pregnancy and occurs more frequently among women with higher FF Cd. The limited number of previous studies characterizing factors predictive of FF metals in humans limits our ability to assess the impact. The small number of participants in our study precludes an assessment for clinical pregnancies among women with biochemical pregnancies, the group 'at risk' for a clinical pregnancy. The results of the clinical pregnancy analysis are thus susceptible to a 'competing risk' from biochemical pregnancy, although a similar pattern for effect estimates suggests no substantial impact.

Passive diffusion of solutes through the blood-follicle-barrier is governed by molecular size, although active processes are also likely involved given the high metabolic rate of the developing follicle [47]. The constituents of FF might thereby vary from blood for some elements and might account for the independence of FF Pb and blood Pb concentrations in the study sample. Recent research indicates that FF element concentrations approach blood concentrations as a follicle grows and matures, yet concentrations may vary substantially during development [23]. Given the variability of FF metal concentrations with follicle size, use of FF from a single follicle in one ovary is likely to introduce exposure misclassification. In fact, our analysis of variability sources suggests substantial roles for factors within woman (i.e., within and between follicles) and for analytic sources, undermining the use of a single specimen to characterize exposures for 'cohorts' of oocytes and embryos. While we are unable to isolate the contribution due to sampling of the right or left ovary, such variability was previously reported for FF cotinine [48], and inter-follicular and inter-ovary variability was recently reported for FF lipoprotein constituents [49].

Exposure measurement misclassification secondary to variability associated with factors within woman in our study was likely further exacerbated by FF metal concentrations measured near the LODs. For example, concentrations of Hg, Cd and Pb are 4.6-fold, 13.5-fold and 5-fold lower in FF than in blood, respectively. A substantial proportion of women have FF Cd below the LOD and effect estimates for Cd have the greatest imprecision; a sub-analysis attributes the majority of FF Cd variability to analytic factors. Among FF values above the LOD, a threshold for confidence in detecting the *presence* of a metal, 73.9 % of Hg, 95.7 % of Cd and 30.4 % of Pb values fall below the limits of quantitation (LOQ), equal to 0.8, 0.07 and 0.11 μg/L, respectively. The LOQ is defined as 10 standard deviations of the pooled follicular fluid and provides a threshold for confidence in quantifying the *absolute value* of a metal. Misclassification of FF Cd is possible as values traverse the LOD up to the LOQ, and may in part account for unanticipated observations. In sensitivity analyses, replacement of values below the LOD with the LOD/2 and with the LOD/$\sqrt{2}$ generate similar though more moderate Cd effect estimates (data not shown). We thus suspect exposure misclassification plays a role in the unanticipated results for FF Cd and pregnancy.

Metals including Hg, Cd and Pb occur in near ubiquitous fashion in the environment and so contamination during FF collection, which employs a surgical grade stainless steel needle, may be important. Whereas the validity of measures for certain elements comprising the latter may be of concern, this does not appear to apply to Hg, Cd and Pb; however, we cannot rule out this possibility. Field blanks were unavailable to our laboratory to assess the possibility for specimen contamination, although an analysis of the cryovials used for specimen storage indicated no substantial contribution. An additional issue relates to the possibility for FF contamination by blood during the invasive aspiration procedure. However, our exclusion of two pink-colored specimens and low values for metals argue against a substantial impact.

Conclusions

Overall, with the exceptions of FF Pb and FF Cd with oocyte fertilization, we find little association between FF metals and IVF outcomes at the trace levels of exposure experienced by study participants. Despite the limitations to this preliminary study, we herein provide one of only a handful of reports in the literature to relate concentrations of non-essential toxic metals in FF to reproductive outcomes. This study of toxic metals in FF and reproductive outcomes is among the first to consider women residing in the U.S. We identify several consistencies with our prior work employing metal concentrations assessed in blood and urine from the same study population, as well as the few prior studies published in this area. We note several important discrepancies as well, possibly attributable to exposure measurement misclassification secondary to biologic variability within woman, concentrations of FF metals occurring in very close proximity to or below the LODs, and unmeasured confounding factors. The results of this study do not corroborate our initial presumption that biomarkers measured from a single FF specimen are necessarily superior to blood and urine for evaluating associations with IVF outcomes among women with 'background' exposures. While inconclusive, the results of this pilot investigation suggest that future work should examine multiple follicles using a 'one follicle-one oocyte/embryo' approach. A larger study is merited to assess more definitively the role trace exposures to toxic metals could play with respect to egg quality in IVF programs.

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