Published in final edited form as:
Reprod Toxicol. 2010 June ; 29(3): 298–305. doi:10.1016/j.reprotox.2010.01.003.

Toxic trace metals and human oocytes during *in vitro* fertilization (IVF)

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Abstract

Trace exposures to the toxic metals mercury (Hg), cadmium (Cd) and lead (Pb) may threaten human reproductive health. The aim of this study is to generate biologically-plausible hypotheses concerning associations between Hg, Cd, and Pb and *in vitro* fertilization (IVF) endpoints. For 15 female IVF patients, a multivariable log-binomial model suggests a 75% reduction in the probability for a retrieved oocyte to be in metaphase-II arrest for each μg/dL increase in blood Pb concentration (relative risk (RR) = 0.25, 95% confidence interval (CI) 0.03–2.50, $P = 0.240$). For 15 male IVF partners, each μg/L increase in urine Cd concentration is associated with an 81% decrease in the probability for oocyte fertilization (RR = 0.19, 95% CI 0.03–1.35, $P = 0.097$). Because of the magnitude of the effects, these results warrant a comprehensive study with sufficient statistical power to further evaluate these hypotheses.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

Mercury (Hg); cadmium (Cd); lead (Pb); oocyte maturation; oocyte fertilization; *in vitro* fertilization (IVF); assisted reproductive technologies (ART); intracytoplasmic sperm injection (ICSI)

1. Introduction

Amassing *in vitro* and *in vivo* evidence suggests that exposure to mercury (Hg) [1], cadmium (Cd) [2] and lead (Pb) [3] are widespread and may threaten human reproductive health. Due to their long half-lives, approximately 45–70 days for Hg [4] and 4–47 years for Cd [2], these elements biomagnify in aquatic and terrestrial food chains [5]. Dietary intake (particularly seafood) is the single greatest non-occupational source of exposure to the organic species of Hg in the U.S. [6]. Shellfish, bivalves such as mussels and oysters in particular, comprise a concentrated source of dietary exposure to Cd [5]. Furthermore, unique populations may be exposed to high concentrations of inorganic Hg salts and/or Pb through non-occupational sources, such as the use of traditional folk remedies [7].

Reproductive outcome data reported by U.S. fertility clinics for 1999–2000 suggests that women of Asian ethnicity experience reduced odds for a clinical pregnancy following a first cycle of *in vitro* fertilization (IVF) compared to Caucasian women [8]. An analogous pattern is evident for the substantial portion of Asian women (~33%) comprising the patient population of our IVF clinic [9]. One possible explanation for these observed discrepancies in IVF success rates by ethnicity is increased exposure to reproductive toxicants including Hg, Cd, and Pb due to differences in fish and shellfish consumption behavior [10,11], as well as the use of traditional herbal remedies [7].

Increasing evidence indicates human reproductive toxicity is associated with prolonged exposure to high doses of metals, including Cd and Pb, in occupational settings [12,13]. However, controversy persists in regard to reproductive toxicity due to the background or ‘trace’ exposures to Hg, Cd and Pb received by most humans. Investigators evaluating populations without documented occupational exposure to these metals have reported reduced fecundity and fertility among women [14–18] and/or men [14,19–22]. However, studies have also reported either no association or an increase of fecundity and/or fertility with metals exposure among women [17,23] or men [15,24,25].

The aim of this preliminary study is to explore potential associations between metals exposures and periconception events in female IVF patients and their male partners without documented occupational metals exposures. Specific testable hypotheses, and methodologic recommendations, will be generated for consideration in a future confirmatory study to evaluate suggested associations. To accomplish this aim, body burdens of Hg, Cd, and Pb indicative of chronic background exposures were measured in female IVF patients and their male partners, and associations with oocyte maturation and fertilization are considered.

2. Methods

2.1 Sample Selection

The sample for this study comprised women and their male partners who were referred to the Center for Reproductive Health of the University of California at San Francisco (UCSF) for infertility treatment. Approximately 400 1st IVF cycles are performed annually in the clinic. The clinic reported an overall oocyte fertilization rate of 65%, implantation rate of 20% and clinical pregnancy rate of 43%, with an average of 3.1 embryos transferred during the study period. Approximately 10% of IVF cycles were cancelled, primarily due to inadequate

follicular response. Fifty-eight female patients and 36 male partners were recruited. Between September 1st, 2007 and August 31st, 2008 prospective IVF patients were identified as potential study participants for the Study of Metals in Assisted Reproductive Technologies (SMART) if it was their 1st IVF attempt at the center. Only those prospective patients undergoing a 1st IVF procedure were recruited to this study in an effort to reduce the introduction of bias that may occur should the experience of an earlier IVF treatment cycle affect the decision to repeat IVF, alter the baseline risk for the reproductive endpoints of interest, or shift the distributions for the exposures of interest [26]. As part of routine treatment, women presenting for IVF receive an initial infertility evaluation including medical and reproductive histories. Men receive an initial baseline clinical evaluation that includes one or more semen analyses to assess volume, sperm concentration, motility and morphology. Informed consent was obtained during the pre-cycle preparation time period from all study participants. The study protocol was approved by the Institutional Review Board of the University of California at San Francisco. Participants completed a questionnaire to ascertain behaviors such as cigarette smoking, use of folk health treatments and seafood consumption and agreed to provide blood and urine specimens for analysis.

2.2 Clinical Protocol

Female study participants underwent gonadotropin-induced ovarian stimulation per clinic protocols during which time serum estradiol measurements and transvaginal ultrasonagraphy were employed to monitor follicular maturation and endometrial development. When a sufficient number of follicles had matured (i.e., ≥17 mm diameter), human chorionic gonadotropin (hCG; 5,000–10,000 IU) was administered subcutaneously and oocytes were retrieved 36 hours later. A fasting whole blood and urine specimen was obtained from women at the time of oocyte retrieval (patients are required to fast prior to oocyte retrieval to facilitate conscious sedation during the procedure). A non-fasting whole blood and urine specimen was obtained from men, when available, on the same day (partners are not required to fast prior to the day of oocyte retrieval). Blood specimens were collected into evacuated 6 mL lavender-top Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ) containing 10.8 mg K_2-EDTA, allowed to stand for 10 minutes, aliquoted into 1.8 mL cryovials and immediately frozen at −80°C. Random urine specimens were collected into urine collection cups, aliquoted into 1.8 mL cryovials and immediately frozen at −80°C. Collected oocytes in metaphase-II (MII) arrest were fertilized by conventional insemination or by intracytoplasmic sperm injection (ICSI) using sperm from male partners retrieved on the day of oocyte retrieval, or a frozen sperm specimen from the male partner or a donor. In cases involving conventional insemination, all collected cumulus masses containing oocytes were inseminated with ~50,000–100,000 sperm. Approximately 16–18 hours following insemination, zygotes were identified by the appearance of two pronuclei.

2.3 Exposure Assessment

Blood and urine specimens were obtained from 58 women and 36 men and analyzed for Hg, Cd and Pb. Eight blood specimens collected from women and three collected from men were not of sufficient volume to permit analysis for Hg and Pb. Three women did not provide sufficient urine specimen volume to permit analysis for Cd [27]. The State of California mandates reporting of all blood Pb test results along with personal identifiers, for blood specimens collected within the state [28]. Since this statute conflicts with the confidentiality requirements for this study, an ‘opt-out’ clause for blood Pb testing was incorporated into the informed consent. Due to confidentiality concerns blood Pb analysis was conducted for only 27 women and 19 men. A complete panel of metals was measured for 25 women and 15 men (i.e., blood Hg and Pb, and urine Cd).

Whole blood and urine specimens were shipped on dry ice in 1.8 mL cryovials to the Trace Elements Section of the Laboratory of Inorganic and Nuclear Chemistry, Wadsworth Center, New York State (NYS) Department of Health (Albany, NY). The laboratory was blinded to clinical data for men and women and IVF endpoints. Blood specimens were analyzed for Hg and Pb using a method optimized for a Perkin Elmer Sciex ELAN DRC II inductively coupled plasma-mass spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT) with dynamic reaction cell technology (DRC-ICP-MS), and equipped with a Burgener Mira Mist® nebulizer (Burgener Research Inc., Mississauga, Ontario, Canada) and a Cinnabar cyclonic spray chamber (Glass Expansion, Pocasset, MA). The ICP-MS was operated according to a previously detailed procedure that is certified and approved for use in NYS [29]. Briefly, blood specimens were diluted 1+49 with a diluent solution containing 0.5% (v/v) double-distilled HNO_3, 25 μg/L rhodium (Rh) and 25 μg/L iridium (Ir) as internal standards, 1 mg/L gold (Au) to control Hg memory effects in the spray-chamber, and 0.005% (v/v) Triton ® X-100 (Dow Chemical Co., Midland, MI). Calibration curves were established using six calibration standards (2–40 μg/L for Hg and 1.7–50 μg/dL for Pb) traceable to reference materials from the National Institute of Standards and Technology (NIST) and spiked with caprine base blood to correct for matrix effects. The three most abundant naturally-occurring Pb isotopes, ^{206}Pb, ^{207}Pb and ^{208}Pb were monitored and their ion signals summed for analytical purposes because of the variation in Pb isotope ratios in nature. We analyzed four levels of internal quality control (IQC) blood materials per run (range 1.1–11.9 μg/L for Hg and 3.3–40.7 μg/dL for Pb). Typical method inter-day precisions reported as coefficients of variation for the lowest concentration IQC were 7% at 1.1 μg/L for Hg and 1% at 3.3 μg/dL for Pb.

The analysis for Cd in urine was carried out with the same ICP-MS instrumentation used for blood metals and a standard method previously described in detail [30]. In brief, urine was diluted 1+19 for analysis with 2% (v/v) double distilled HNO_3, 0.005% Triton® X-100 as a surfactant, 1 mg/L Au, and 10 μg/L Rh as an internal standard under Class 100 clean conditions. In addition to monitoring ^{114}Cd, we also measured the element molybdenum (Mo) to monitor molybdenum oxide (MoO) polyatomic interference at m/z 114, that can occur when urine Cd levels are close to background levels and urine Mo levels are very high [31]. Standard curves were generated for each metal using a minimum of six calibration standards (range 0.8–40 μg/L). Standards were traceable to NIST reference material. We analyzed four levels of IQC urine materials (range 0.39–15.1 μg/L) and three levels of external reference materials (range 1.8–5.9 μg/L). The intra-day between run coefficients of variation was 3% at 0.39 μg/L for the lowest concentration IQC and 3% at 1.8 μg/L for the lowest concentration external reference material.

Method specific limits of detection (LOD) were defined as three times the standard deviation of concentrations measured in matrix blanks for 10 independent analyses: 0.2 μg/L for blood Hg, 0.17 μg/dL for blood Pb, 0.02 μg/L for urine Cd and 2.5 μg/L for urine Mo. For statistical purposes concentrations were reported without regard to LODs to preclude the introduction of bias that has been demonstrated when censoring values below the detection limit [32]. Urine values were corrected for creatinine only for reporting and to facilitate comparison to previously published values. During statistical analysis, Cd values were employed without a creatinine correction to preclude the introduction of bias that may occur when using the traditional procedure [33].

2.4 Statistical Analysis

Distributions were characterized for metal concentrations in women and men, and for covariates. Non-parametric methods were employed to evaluate bivariate associations due to lack of normality. Using study participant as the unit of analysis, Spearman rank correlation coefficients were employed to assess bivariate associations among continuous and ordinal

variables, including measured metals and covariates, and Wilcoxon rank-sum and Kruskall-Wallis testing was employed to evaluate metals concentrations by categorical covariates. The proportion of mature oocytes collected 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. Consistent with the hypothesis generating nature of this preliminary study, statistical significance was defined as $P < 0.10$ for a two-tailed test and no adjustments were made to accommodate type-1 error inflation consequent to multiple statistical tests. SAS v. 9.1.3 (SAS Institute, Cary, NC), was used for all statistical analyses.

Using the oocyte as the unit of analysis, multiple log-binomial regression [34] was employed to assess associations between oocyte maturity (metaphase-II arrest, no/yes), oocyte fertilization (zygote, no/yes) and the concentrations of metals adjusting for age as a continuous variable [35], cigarette smoking as the dichotomous variable “never”/”ever”[36], and race/ethnicity as the dichotomous variable “other”/”Asian” [37]. These covariates were selected for inclusion in the multiple log-binomial regression models using literature review followed by incorporation into directed acyclic graphs (DAGs). Directed acyclic graphs employ causal graphing theory to identify a minimally sufficient set of variables with which to control confounding under a postulated causal pathway [38]. For two men with missing race/ethnicity data we assumed the same/race ethnicity as the female partner (i.e., “other”). Separate models were generated for women and men; single metal models (SMM) for each metal as the sole predictor of interest, as well as multiple metal models (MMM) including all metals as predictors of interest. Creatinine concentration was entered as a covariate only for those models including urine Cd as a predictor. Generalized estimating equations (GEE) were used to provide robust standard errors [39] given the inherent clustering of endpoints by subject.

3. Results

3.1 Demographic and Clinical Factors

Distributions for demographic and clinical factors describing female patients and their male partners are presented in Table 1. Women with a median age of 36 years (range 28 to 44) are mostly White (68.4%), although many are Asian (27.6%). Most women were diagnosed with “unexplained” (32.8%) or “male factor” (22.4%) infertility; the latter being evident from the high proportion of ICSI procedures (63.8%). More than half of women (51.7%) received a down-regulated gonadotropin releasing hormone (GnRH) agonist stimulation protocol. Body mass index (BMI), calculated as body mass divided by the square of height, is generally low with the 75^{th} %tile equal to 25 kg/m^2; however the maximum value approaches 46 kg/m^2. A minority of women report having ever smoked cigarettes (17.2%, n = 5 at the time of this study). Men are older than women (median = 38 years), and comprise a smaller proportion of Asian race/ethnicity (14.7%) but a larger proportion of ever cigarette smokers (19.4%, n = 4 at the time of this study). A median of 11 oocytes (range 2–39) were collected from each woman on the day of retrieval. On average 77% of oocytes collected from ICSI cases were in MII-arrest. Sixty-four percent of oocytes collected were fertilized for both ICSI and conventional insemination cases. With one exception, no important bivariate associations are detected for women and men, between the demographic and clinical factors listed in Table 1, and proportions of oocytes in MII-arrest or of oocytes fertilized. A greater proportion of oocytes were fertilized using ICSI (median = 0.76) compared to conventional insemination (median = 0.56) procedures ($P = 0.022$).

3.2 Distributions and Bivariate Associations for Measured Metals

Table 2 presents the distributions for metals measured in blood and urine specimens. Median (range) blood concentration for female Hg and Pb are 2.85 μg/L (0.28–8.77) and 0.77 μg/dL (0.34–1.50), respectively and all values exceed the LODs. Median (range) urine Cd concentration for women is 0.30 μg/g creatinine (0.04–0.98) and 95% of values exceed the LOD. No correlation is suggested between blood concentrations of Hg and Pb measured in women (r = −0.11, $P = 0.579$). After adjusting for urine creatinine there is also no correlation suggested for female urine Cd with blood Pb (r = 0.02, $P = 0.911$), however the possibility for a correlation between urine Cd and blood Hg is indicated (r = 0.22, $P = 0.129$).

As indicated by Table 2, the median (range) blood concentrations for male partner Hg and Pb are 4.15 μg/L (0.57–17.40) and 1.32 μg/dL (0.55–3.67), respectively; all values exceed LODs. Median (range) male Cd urine concentration is 0.13 μg/g creatinine (−0.07–0.51) with the majority of values (55.2%) above the LOD. No correlation is suggested between blood concentrations of Hg and Pb measured in men (r = −0.04, $P = 0.863$), urine Cd and blood Pb concentration adjusted for urine creatinine (r = 0.26, $P = 0.361$), or urine Cd and blood Hg adjusted for urine creatinine (r = 0.06, $P = 0.761$).

As described in Table 2 the concentrations of blood Hg ($P = 0.038$) and blood Pb ($P = 0.002$) are lower among women than among men. However, the association between metals and gender is reversed for concentrations of urine Cd for which values among women are greater than values among men ($P = 0.003$). Although there are differences between blood and urine metal levels between women and men there is a positive correlation between them for blood Hg (r = 0.59, $P = 0.001$) and blood Pb (r = 0.48, $P = 0.085$). Furthermore, concentrations of blood Hg among women demonstrate a positive unadjusted correlation to the proportion of oocytes collected in MII-arrest (r = 0.45, 95%CI 0.11–0.69, $P = 0.011$).

3.3 Multivariable Log-binomial Analysis

The results of the multivariable log-binomial regression of oocyte maturity on metals measured in female ICSI patients are presented in Table 3, adjusted for age, race/ethnicity and cigarette smoking. In a SMM including blood Hg as the only predictor of interest, a one μg/L increase is associated with a 23% increase in the probability for a collected oocyte to be in MII-arrest (risk ratio (RR) = 1.23, 95%CI 0.97–1.55; $P = 0.082$). In contrast, a SMM including female blood Pb as the only predictor of interest indicates a 46% decrease in the probability for a collected oocyte to be in MII-arrest for each one μg/dL increase in concentration (RR = 0.54, 95%CI 0.31–0.93, $P = 0.027$). The MMM, in which all metals measured in women are entered as predictors of interest, suggests a 75% decrease in the probability for a collected oocyte to be in MII-arrest for each one μg/dL increase in blood Pb concentration (RR = 0.25, 95%CI 0.03–2.50, $P = 0.240$). The sample size for the MMM is however quite limited (n = 15) and consequently the confidence interval for the adjusted effect of blood Pb on the probability for MII-arrest is wide. For all SMMs female age is an inverse predictor of oocyte maturity (RR = 0.86–0.95, $P < 0.0001$ to $P = 0.078$), whereas Asian race/ethnicity is a positive predictor in the SMM for Pb (RR = 3.89, $P = 0.092$) and in the MMM (RR = 6.95, $P = 0.230$).

The results of the multivariable log-binomial regression of oocyte fertilization on metals measured in female patients and their male partners, including cases of ICSI and conventional insemination, are presented in Table 4, adjusted for age, race/ethnicity and cigarette smoking. For women, urine Cd demonstrates a consistent and positive association with oocyte fertilization in the SMM and the MMM (RR = 1.27 and RR = 1.41, respectively). Asian race/ethnicity is a positive predictor of fertilization in the SMM model in which Pb (RR = 1.46, $P = 0.001$) is the only predictor of interest, as well as in the MMM including all metals measured in women as predictors of interest (RR = 1.29, $P = 0.035$). The SMM in which male blood Pb

concentration is the sole predictor of interest suggests a 13% increase in the probability for oocyte fertilization for each μg/dL increase in concentration (RR = 1.13, 95%CI 1.01–1.27, $P = 0.039$). The MMM for men, including all measured metals as predictors of interest suggests an 81% decrease in the probability for oocyte fertilization for each μg/L increase in urine Cd concentration (RR = 0.19, 95%CI 0.03–1.35, $P = 0.097$). Asian race/ethnicity among men is a positive predictor of oocyte fertilization in those SMMs in which Hg (RR = 1.31, $P = 0.007$) and Cd (RR = 1.35, $P = 0.004$) are each the only predictor of interest. Cigarette smoking among men is a positive predictor of oocyte fertilization in the SMM for Pb (RR = 1.38, $P = 0.0003$) as well as in the MMM, including all measured metals as predictors of interest (RR = 1.60, $P = 0.001$).

4. Discussion

The results of this preliminary study suggest that trace exposures to toxic metals might influence oocyte maturation and fertilization during IVF, as assessed by the proportion of collected oocytes in MII-arrest and the proportion of zygotes formed *in vitro*. Following adjustment for blood Hg, urine Cd and creatinine, age, race/ethnicity and cigarette smoking a 75% decrease in the probability of collecting an oocyte in MII-arrest is suggested for each μg/dL increase in blood Pb concentration measured in women; however due to the small sample size of our study, the confidence interval for this suggested effect is wide. Bivariate analysis and multivariable analysis using a SMM initially suggest a positive association between female blood Hg concentration and the probability for retrieval of an oocyte in MII-arrest, however this association is diminished by adjustment for Cd and Pb concentrations using a MMM. In contrast, using the MMM approach an 81% decrease in the probability for successful oocyte fertilization is suggested for each μg/L increase in the concentrations of urine Cd measured from men. Urine Cd measured in specimens collected from women may be associated with a 41% increase in the probability for successful oocyte fertilization, following adjusting for other metals and confounding variables. However, as the sample size for this preliminary study was highly limited the adjusted effect of urine Cd on oocyte fertilization has wide confidence intervals, and thus sampling error may explain all or part of this or the other observations.

4.1 Comparison of Measured Metals to Reported Reference Values

Concentrations of blood Hg measured among participants in this study are greater than those reported for the 2003–2006 U.S. population [40]. Median blood Hg measured in female IVF patients (2.85 μg/L, 95%CI 2.06–3.42) and their male partners (4.15 μg/L, 95%CI 2.48–5.03) exceed those reported for U.S. women (0.80 μg/L, 95%CI 0.79–0.90) and men (0.80 μg/L, 95%CI 0.71–0.90). These elevated blood Hg concentrations are consistent with the high proportion of Asian subjects in our sample (i.e., 27.6%) as compared to the general U.S. population (i.e., 4%) [41]. The median Hg concentration among Asian women and men participating in this study (4.01 and 5.12 μg/L blood, respectively) exceeds that for non-Asian study participants (2.64 and 3.21 μg/L blood, for women and men respectively). A recent Community Health and Nutrition Examination Survey (CHANES) conducted in NY City from 2002–2004 reported significantly higher blood Hg concentrations compared to the U.S. population [42]. In that study, Asian subjects had higher geometric mean blood Hg levels compared to the general NY City population; this difference was attributed to dietary sources of Hg. The positive correlation between female and male blood Hg concentrations in the current study suggests a common exposure pathway, likely dietary [10,43]. Whereas blood Hg concentrations among men significantly exceed those measured for women in this study, we cannot assess whether this reflects differences in exposure or sampling variability *vis a vis* non-fasting and fasting specimens.

Concentrations of urine Cd measured among subjects in the current study resemble those reported for the 2001–2002 U.S. population [44]. The median concentration for women in this study (0.30 μg/g creatinine, 95%CI 0.21–0.36) is similar to that reported for U.S. females (0.26 μg/g creatinine, 95% CI 0.23–0.30) whereas the median concentration for the male partners (0.13 μg/g creatinine, 95%CI 0.11–0.16) is somewhat lower than that reported for U.S. males (0.17 μg/g creatinine, 95%CI 0.16–0.18). Women demonstrate greater urine Cd values than men; a difference that persists when restricted to 'never smokers' and is consistent with previous reports [5,44]. Men demonstrate a substantial proportion of urine Cd values below the LOD (44.8%) thus this result should be interpreted with caution. In our study sample, self-reported 'ever smokers' demonstrate higher median urine Cd concentrations (0.22 μg/g creatinine) than 'never smokers' (0.18 μg/g creatinine).

Concentrations of blood Pb measured among subjects in this study are similar to or lower than those reported for the 2001–2002 U.S. population [44]. The median blood Pb concentration measured among women in this study (0.77 μg/dL, 95%CI 0.64–1.04) is lower than was reported for U.S. women (1.10 μg/dL, 95%CI 1.10–1.20), whereas that for men (1.32 μg/dL, 95%CI 1.04–2.11) is similar (1.70 μg/dL, 95%CI 1.70–1.80). The Pb concentrations measured in this study are substantially lower than median values reported for women and men in the general U.S. population in 1976 (11.0 and 15.0 μg/dL blood, respectively), prior to the gradual phase-out of tetraethyl Pb as an 'anti-knock' gasoline additive which began in that year [45]. Concentrations of Pb measured in this study are also lower than those measured for women and men in the general U.S. population in 1991 (1.4 and 2.4 μg/dL blood, respectively), shortly following the 1986 completion of the U.S. phase-out of tetra-ethyl Pb as a gasoline additive [45]. Moreover, the maximum measured value for blood Pb in this study (i.e., a male with 3.67 μg/dL) is lower than the current 10 μg/dL 'level of concern' promulgated by the U.S. Centers for Disease Control and Prevention (CDC), as well as the current 40 μg/dL blood occupational exposure threshold for notification and medical examination set by the U.S. Occupational Safety and Health Administration (OSHA) [3]. Thus, the concentrations of Pb measured in this study are very low compared with historical values as well as in comparison to current regulatory thresholds of concern for human health effects. The positive correlation between Pb concentrations in women and men suggests a common source of exposure, despite the greater values detected for the latter

4.2 Associations between Metals and Oocyte Maturation

We here report evidence of a potential inverse association between blood Pb concentrations measured from female IVF patients and the probability of retrieving a mature oocyte (i.e., MII-arrest). The progression of mammalian germinal vesicle (GV) oocytes from late prophase (in which they arrest *in utero*) to MII (in which they arrest until fertilization) is initiated by the mid-cycle surge in luteinizing hormone concentrations [46], or human chorionic gonadotrophin (hCG) during assisted reproduction. Progression is contingent upon the concentrations of several signaling factors including cyclic adenosine 3′, 5′-monophospohate (CAMP) [47]. Calcium (Ca^{2+}) dependent protein kinase enzymes, including mitogen-activated protein kinase (MAPK), play critical roles in the orchestration of mammalian oocyte progression from the GV stage to the MII-arrest stage [48]. Ionized Pb^{2+} has been demonstrated to supplant Ca^{2+} and thereby moderate protein kinase activity [49] and possibly oocyte progression from GV to MII-arrest.

Recent evidence indicates Pb^{2+} inhibits the GV to MII-arrest transition via disruption of protein kinase activity [50]. Although we observed no such indication in the current study, similar observations are reported for Cd in which exposure significantly reduced the maturation rate of cultured ovine GV stage oocytes [51]. Moreover, several investigators have reported detectable concentrations of Pb and/or Cd in human follicular fluid specimens [52–55].

Unfortunately, follicular fluid specimen analysis was not available to us at the time of this study however we plan on conducting these analyses as part of a future study. We are unaware of prior studies considering human oocyte maturity as an endpoint in association with exposure to metals although prior research indicates reduced fertility in association with female Pb exposure [17,18,52,56].

4.3 Association between Metals and Oocyte Fertilization

Our study results do not suggest associations between female trace exposure to toxic metals and oocyte fertilization among IVF patients. It is interesting however to note the positive point estimate detected in the MMM describing a 41% increased probability for oocyte fertilization for each unit increase in urine Cd concentration (RR = 1.41), irrespective of statistical precision (95%CI 0.62–3.17). Investigators have previously indicated positive associations between Cd exposure and oocyte fertilization in women receiving IVF, although these prior studies employed follicular fluid to estimate exposure [17,54]. As noted above, follicular fluid was unfortunately unavailable at the time of this study and thus a direct comparison of results is not currently feasible. The absence of an association between female blood Hg concentrations and oocyte fertilization is consistent with the results of a prior study in which no such association was suggested for women undergoing IVF (odds ratio (OR) = 0.70, 95%CI 0.19–2.57), and for whom a similar median blood Hg concentration (3.2 μg/L; range <0.3–30.4) was reported [17].

These study results raise the possibility of an inverse association between urine Cd concentrations in male partners of female patients undergoing IVF and oocyte fertilization. In contrast to the aforementioned positive association indicated for urine Cd concentrations among women and oocyte fertilization, each unit increase in male urine Cd concentration is associated with an 81% decrease in the probability for zygote formation (RR = 0.19), with a comparatively narrow confidence interval (95%CI 0.03–1.35). Several researchers have previously reported inverse associations between body burdens of Cd in the male partner and fertility [22,57] whereas others have reported no such associations [24,58]. These prior studies employed seminal plasma to estimate exposure, which was unfortunately unavailable to us during this preliminary study. In similar fashion several investigators have previously reported inverse associations between seminal plasma Pb and fertility among individuals conceiving spontaneously [19] or using assisted reproductive technologies [20,21]. We are unable to directly evaluate our results in the context of this prior literature due to the unavailability of metals data in seminal plasma. However, our study results conflict with those reported for a Chinese IVF population [14], in which the average blood Hg concentration measured for the male partner of infertile couples (8.14 μg/L) exceeded (P = 0.03) that of the male partners of fertile couples (6.23 μg/L). Male partners in our sample demonstrate lower average blood Hg concentrations (4.34 μg/L) than male partners in that prior study.

4.4 Limitations and Strengths of the Study

The results of this study are only preliminary in nature, providing hypotheses for further evaluation in a future comprehensive investigation with sufficient statistical power. Several critical limitations restrict interpretation of the results from this preliminary investigation and thus these should be interpreted with caution and only be considered suggestive. A small number of participants were considered in this study, restricting the statistical power for detecting subtle associations between metals and IVF endpoints and furthermore limiting our ability to simultaneously consider exposures in female patients and their male partners. Given the couple-level nature of reproduction the simultaneous consideration of patient and partner exposures is highly desirable [59]. Furthermore, due to the limited resources available to us for the conduct of this preliminary study only blood and urine measures of metals exposure were considered, whereas these matrices provide an approximation of the internal dose

resulting from chronic exposure to the metals of interest, it would appear to be more desirable to employ follicular fluid and semen to more closely approximate biologically effective/target organ doses. In addition, using a larger sample, in the future, we intend on assessing the possibility that associations between metals exposures and IVF endpoints may be modified by infertility diagnosis.

Our recruitment of only female patients and their male partners undergoing their1st IVF cycle at an IVF clinic limited the sample size for this preliminary study. However, this approach minimizes the introduction of bias that may result should prior IVF experience be associated with the study endpoints of interest or the exposures of interest [26]. Moreover, our recruitment of only IVF couples to this study, excluding couples conceiving unassisted, is necessary to preclude the introduction of bias that might result from inherent differences between infertile and fertile couples, such as behaviors leading to metals exposure [60].

In spite of the restricted sample size and limited resource availability, the use of the oocyte as the unit of measure during multivariable analysis provided multiple outcomes per subject (i.e., 2–39 oocytes per patient) which inflated study power sufficiently to facilitate the convergence of log-binomial models while adjusting for important covariates. Generalized estimating equations (GEE) were used to accommodate these multiple outcomes and provide robust standard error estimates. We employed a prospective study design facilitating the capture of peri-conceptional events (i.e., oocyte fertilization) and ensuring temporality (i.e., exposure preceded outcome). Couples employing assisted reproductive technologies such as IVF are subject to intense data collection and follow-up, spanning the interval between folliculogenesis and the 1st trimester of gestation. Thus IVF female IVF patients and their male partners are uniquely suited to the prospective study of environmental exposures and periconceptional events, the latter of which are excluded by the nature of retrospective studies in populations conceiving unassisted [61].

It has been reported that when very low concentrations of Cd in urine are measured by ICP-MS in the presence of very high Mo concentrations, a polyatomic interference from molybdenum oxide (MoO) can produce a small positive bias in very low concentration urine Cd results [31]. This resulted in a mathematical correction being applied to the 2000–2001 National Health and Nutrition Examination Survey (NHANES) data for urine Cd. In this study, urine Mo concentrations were also measured by ICP-MS to provide for a similar correction should it be deemed necessary. Interference by MoO at m/z 114 could be problematic for populations with high Mo exposure coincident to low Cd exposure. The urine Cd values measured in this study are similar to or below those reported for the U.S. population (i.e., NHANES 2001–2002), values that were corrected for MoO interference. Furthermore, urine Mo values measured in this study are similar to those reported for the 2001–2002 U.S. population by NHANES (data not shown). These observations indicate that a MoO correction is not necessary for the purposes of this study.

The metals measured in blood and urine during this study occur ubiquitously in the environment, raising the possibility of specimen contamination during the collection and storage period. Lavender-top Vacutainer tubes containing potassium EDTA manufactured by Becton Dickinson Co. are routinely used in biomonitoring studies to determine blood metals (Pb, Cd, Hg) at low concentrations [29,42]. Although field blanks were unavailable to our laboratory to confirm the absence of systematic contamination, in blood collection tubes or urine collection cups, a subsequent evaluation of the cryovials used to store blood and urine specimens, coupled with a review of the analytical data produced indicate no substantial contamination occurred. Based on our prior experience, the screening of specimen storage containers, and the concentrations of blood/urine metals in our sample relative to that reported for the U.S. we have no compelling evidence to suggest that prior sample collection container

contamination explains the associations here suggested between Pb concentrations and oocyte maturity or between Cd concentrations and oocyte fertilization.

4.5 Conclusions

Despite the limitations to the current study we report herein several data-driven biologically-plausible hypotheses for confirmation or refutation in a future, larger and more comprehensive study of trace exposures to toxic metals and IVF endpoints. Although no associations are suggested between blood Hg concentration and oocyte maturity or fertilization by this study, we here raise the possibility that Pb might affect maturation of oocytes during IVF and that female and male Cd exposure might both play important, although contradictory roles in oocyte fertilization during IVF. These hypotheses are of particular public health relevance given that the measured concentrations of urine Cd and blood Pb in this study are similar to or lower than, respectively; those reported for the general U.S. population. These provocative but preliminary results merit a larger comprehensive study with sufficient sample size to permit the simultaneous consideration of female patient and male partner exposures with adjustment for a wide spectrum of confounding variables, consideration of effect modification by clinical factors, sufficient statistical power to confirm or refute the suggested associations, as well as consideration of biologically relevant specimens for modeling exposure, specifically follicular fluid and semen. These results raise questions whose solutions will begin to address a critical knowledge gap regarding the impact of chronic exposure to trace concentrations of non-essential metals on fertilization and very early pregnancy among couples undergoing IVF-treatment.

Acknowledgments

We would like to thank Chris D. Palmer, Ph.D, for providing expert input regarding analysis of metals in blood specimens and to extend our gratitude to the study participants whose generosity made this study possible. This work was not supported by any external grant funding. Institutional discretionary research funds available to Drs. Bloom and Fujimoto were used to support this work. This research was also supported in part by the Intramural Research Program of the NIH, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development.

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Table 1

Distribution of demographic and clinical variables in female patients and their male partners; the Study of Metals and Assisted Reproductive Technology (SMART).

Demographic & Clinical Variables	N	Mean	SD	Min.	25th %tile	Median	75th %tile	Max.
Female patients								
Age (years)	58	35.8	3.9	28.0	32.0	36.0	38.0	44.0
Body mass index (kg/m^2)	55	24.3	5.0	17.9	21.2	23.3	25.3	45.6
Ever cigarette smoker (%) [a]	10	17.2	-	-	-	-	-	-
Race/Ethnicity (%)	58	-	-	-	-	-	-	-
Not Asian	42	72.4	-	-	-	-	-	-
Asian	16	27.6	-	-	-	-	-	-
Urine creatinine (mg/dL)	55	131.1	88.7	24.0	60.0	113.9	189.5	415.3
Primary infertility diagnosis (%)								
Unexplained	19	32.8	-	-	-	-	-	-
Male factor	13	22.4	-	-	-	-	-	-
Diminished ovarian reserve	10	17.2	-	-	-	-	-	-
Tubal factor	7	12.1	-	-	-	-	-	-
Endometriosis	3	5.2	-	-	-	-	-	-
Anovulation	3	5.2	-	-	-	-	-	-
Other	3	5.2	-	-	-	-	-	-
Intracytoplasmic sperm injection (%)	37	63.8	-	-	-	-	-	-
Male partners								
Age (years)	36	38.4	4.3	31.0	35.0	38.0	41.5	48.0
Ever cigarette smoker (%) [a]	7	19.4	-	-	-	-	-	-
Race/Ethnicity (%) [b]	36	-	-	-	-	-	-	-
Not Asian	29	85.3	-	-	-	-	-	-
Asian	5	14.7	-	-	-	-	-	-
Urine creatinine (mg/dL)	36	125.2	71.8	14.1	69.7	108.6	181.5	253.0
IVF endpoints								
Number of oocytes collected	58	13.1	7.8	2.0	8.0	11.0	18.0	39.0
Proportion mature oocytes	37	0.77	0.22	0.20	0.60	0.83	0.95	1.00

Demographic & Clinical Variables	N	Mean	SD	Min.	25th %tile	Median	75th %tile	Max.
Proportion fertilized oocytes	55	0.64	0.27	0.00	0.50	0.68	0.83	1.00

[a] Self-reported active or past cigarette smoking;

[b] n = 2 missing values; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; Max, maximum value; Min, minimum value; SD, standard deviation.

Table 2

Distribution of metals concentrations measured on the day of oocyte collection in female patients and their male partners; the Study of Metals and Assisted Reproductive Technology (SMART).

Metals	n	Mean	SD	Min	25th %tile	Median	75th %tile	Max	%>LOD
Female patients									
Blood mercury (μg/L)	50	3.07	1.95	0.28	1.69	2.85	3.90	8.77	100.0
Urine cadmium (μg/g creatinine)	55	0.30	0.17	0.04	0.16	0.30	0.40	0.98	94.8
Blood lead (μg/dL)	27	0.82	0.32	0.34	0.55	0.77	1.05	1.50	100.0
Male partners									
Blood mercury (μg/L)	33	4.34	3.15	0.57	2.05	4.15	5.37	17.40	100.0
Urine cadmium (μg/g creatinine)	36	0.15	0.11	−0.07 [a]	0.10	0.13	0.18	0.51	55.2
Blood lead (μg/dL)	16	1.50	0.80	0.55	0.99	1.32	1.93	3.67	100.0

[a] Negative value due to no censoring of values below the LOD.

ICSI, intracytoplasmic sperm injection; LOD, limit of detection; Max, maximum value; Min, minimum value; SD, standard deviation.

Table 3

Multiple log-binomial regression of oocyte maturity on concentrations of metals measured in female ICSI patients, with generalized estimating equations used to generate robust standard errors; the Study of Metals and Assisted Reproductive Technology (SMART).

Predictor Variables	n [a]	RR [b]	95% CI		RR [c]	95% CI	
			Low	High		Low	High
Blood mercury (μg/L)	31	1.23 *	0.97	1.55	0.79	0.32	1.91
Urine cadmium (μg/L) d	36	1.19	0.24	6.01	1.05	0.11	10.27
Blood lead (μg/dL)	16	0.54 **	0.31	0.93	0.25	0.03	2.50

[a] Sample size varies for single metal models due to missing values for metals concentrations;

[b] Relative risk (RR) for MII-arrest as a function of concentrations of a single measured metal adjusted for age, cigarette smoking, race/ethnicity and urine creatinine (mg/dL) for cadmium;

[c] n = 15, relative risk for MII-arrest as a function of concentrations of all measured metals adjusted for age, cigarette smoking, race/ethnicity and urine creatinine (mg/dL);

* $P \leq 0.10$;

** $P \leq 0.05$.

CI, confidence interval; ICSI, intracytoplasmic sperm injection.

Table 4

Multiple log-binomial regression models of oocyte fertilization *in vitro* on concentrations of metals measured in female patients and their male partners, with generalized estimating equations used to generate robust standard errors; the Study of Metals and Assisted Reproductive Technology (SMART).

Predictor Variables	n [a]	RR [b]	95% CI Low	95% CI High	RR [c]	95% CI Low	95% CI High
Patients							
Blood mercury (μg/L)	50	1.01	0.96	1.06	0.99	0.93	1.07
Urine cadmium (μg/L) [d]	55	1.27	0.83	1.95	1.41	0.62	3.17
Blood lead (μg/dL)	27	0.97	0.66	1.43	1.09	0.72	1.65
Partners							
Blood mercury (μg/L)	33	0.97	0.92	1.03	0.97	0.91	1.03
Urine cadmium (μg/L) [d]	36	0.88	0.47	1.64	0.19 *	0.03	1.35
Blood lead (μg/dL)	16	1.13 **	1.01	1.27	1.08	0.97	1.21

[a] Sample size varies for single metal models due to missing values for metals concentrations;

[b] Relative risk (RR) for a zygote as a function of concentrations of a single measured metal adjusted for age, cigarette smoking, race/ethnicity and urine creatinine (mg/dL) for cadmium;

[c] n = 25 patients, n = 15 partners, relative risk for a zygote as a function of concentrations of all measured metals adjusted for age, cigarette smoking, race/ethnicity and urine creatinine (mg/dL);

* $P \leq 0.10$;

** $P \leq 0.05$.

CI, confidence interval