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# Toxic trace metals and embryo quality indicators during *in vitro* fertilization (IVF)

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ABSTRACT

Trace exposures to the toxic metals mercury (Hg), cadmium (Cd) and lead (Pb) may interfere with *in vitro* fertilization (IVF). The aim of this study is to explore biologically plausible hypotheses concerning associations between metals and embryo quality indicators during IVF. For 24 female patients, a multivariable ordinal logistic regression model suggests a 75% reduction in the odds for higher embryo cell cleavage per μg/dL increase in blood Pb (adjusted odds ratio (aOR) 0.25, 95% confidence interval (CI) 0.07–0.86). For 15 male partners, each μg/L increase in blood Hg (aOR 0.60, 95% CI 0.45–0.79) and μg/dL increase in blood Pb (aOR 0.58, 95% CI 0.37–0.91) is associated with a decrease in the analogous odds. Embryo fragmentation is reduced by higher blood Hg (aOR 0.85, 95% CI 0.72–1.00), but increased by higher blood Pb (aOR 1.47, 95% CI 1.11–1.94) in men. The magnitude of these suggested effects warrants confirmation in a larger study.



## 1. Introduction

The non-essential and toxic metals mercury (Hg), cadmium (Cd) and lead (Pb) are widespread in the environment resulting in ubiquitous human exposure [1]. These elements are persistent and tend to accumulate and biomagnify in aquatic and terrestrial food chains. Frequently ingested in trace concentrations over extended periods of time, Hg, Cd and Pb may pass into cells [2], where they increase oxidative damage by depleting available anti-oxidant enzymes [3]. Moreover, divalent metals including Hg, Cd and Pb bind the estrogen receptor and may interfere with intra-cellular sex-steroid hormone signaling pathways [4–6].

Several investigators have described adverse human reproduction outcomes following the exposure of women and men to high concentrations of toxic metals, such as those encountered in occupational settings or industrial accidents [7,8]. More frequently, however, humans are exposed to trace concentrations of toxic metals through dietary sources or airborne pollution [1]. Potential associations between trace toxic metals exposures and reproductive endpoints remain controversial, although an increasing body of literature suggests that infertile populations, including those undergoing *in vitro* fertilization (IVF), may be at an increased risk [9–13].

Reproductive toxicity resulting from long term exposure to trace concentrations of toxic metals may interfere with the success of IVF procedures [8], a possibility underscored by their presence in reproductive tissues collected from women [10,13,14] and men [15–17]. In this paper we expand upon our prior report describing preliminary associations between trace metal exposures and oocyte maturation and fertilization during IVF [12], by considering the subset of study participants for whom embryos were generated. Here we describe preliminary associations between blood and urine concentrations of Hg, Cd and Pb measured in female patients and their male partners, and indicators of embryo quality during IVF.

## 2. Methods

### 2.1. Sample selection and clinical protocol

Sample selection and the clinical protocol for this study were previously reported in detail [12]. In brief, 58 female patients and 37 male partners undergoing a first IVF cycle at the University of California at San Francisco (UCSF) Center for Reproductive Health (San Francisco, CA, USA), between September 1st 2007 and August 31st 2008, were recruited into the Study of Metals and Assisted

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Reproductive Technologies (SMART). Approximately 50% of women invited agreed to participate. Routine treatment included a baseline infertility evaluation for women and men comprising documentation of ovulation, verification of normal uterine and tubal patency, and one or more semen analyses. Women underwent gonadotropin-induced ovarian stimulation per clinic protocol. When a minimum of 2 follicles ≥17 mm diameter was identified ultrasonographically, human chronic gonadotropin (hCG) was administered and oocytes retrieved 36 h later. Fasting whole blood and urine specimens were collected from female patients at the time of oocyte retrieval, and non-fasting whole blood and urine specimens were collected from male partners, when available, on the same day. Collected oocytes in metaphase-2 arrest (MII) were fertilized by intracytoplasmic sperm injection (ICSI) or conventional insemination using fresh sperm from male partners or frozen sperm from a male partner or donor. Total motile count (TMC) was calculated by total sperm concentration (million/mL) × volume of semen (mL) × proportion of motile sperm (%). Zygotes were identified by the appearance of two pronuclei (2PN) approximately 16–18 h following insemination. The study protocol was approved by the UCSF Committee for Human Research.

### 2.2. Exposure assessment

Blood and urine specimens stored at −80 °C were transported on dry ice to the Trace Elements Section of the Laboratory of Inorganic and Nuclear Chemistry, Wadsworth Center, New York State (NYS) Department of Health (Albany, NY, USA). Blood and urine specimens for 54 women and 36 men were analyzed for Hg, Cd, and Pb as previously described [12]; one additional male did not provide urine. In brief, blood specimens were analyzed for Hg and Pb using a Perkin Elmer Sciex ELAN DRC II inductively coupled plasma-mass spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with dynamic reaction cell technology (DRC-ICP-MS) [18], and urine specimens were analyzed for Cd [19]. Limits of detection (LOD) were 0.20 μg/L for blood Hg, 0.17 μg/dL for blood Pb, and 0.02 μg/L for urine Cd. For statistical purposes no censoring of concentrations below the LODs was implemented [20]; machine-read values were analyzed as reported. For reporting purposes urine Cd concentrations were reported as μg/g creatinine.

### 2.3. Study endpoints

Fifty-four women and 36 male partners with at least one 2PN zygote and biospecimen measurement for at least one metal comprise the sample for the current study. A smaller number of participants had measurements for all metals. Embryo quality assessments for study participants were made on the day of transfer by experienced embryologists blinded to exposure data. A single embryologist examined all embryos produced by a couple. Embryo cell number (ECN), a positive predictor for IVF success [21], characterizes cleavage (i.e., growth) rate and is defined as the number of blastomeres counted on the day of embryo transfer. Embryo fragmentation score (EFS), an inverse predictor for IVF success [22], was assessed approximately 48 h following fertilization and characterizes the proportion of lost cell volume to enucleate subdivisions and observed as membrane blebbing. Our lab defined EFS according to an ordinal scale: Grade 1, 0% fragmentation; Grade 2, 1–10% fragmentation; Grade 3, 11–25% fragmentation; Grade 4, 26–50% fragmentation; and Grade 5, ≥51% fragmentation.

### 2.4. Statistical analysis

Distributions were characterized for metal concentrations and covariates for women and men. Using the study participant as the unit of analysis we derived average, or 'embryo cohort' ECN and EFS scores, defined as the mean of individual embryo scores traced back to a participant. At this participant-level of observation non-parametric methods including Spearman correlations, Wilcoxon-rank sum tests and Kruskal–Wallis tests were employed to evaluate bivariate associations due to lack of normality.

Using the embryo as the unit of analysis, multivariable ordinal logistic regression models with selected predictors and covariates were employed to estimate the association between exposure and individual embryo outcomes. We were unable to normalize endpoint distributions to meet the assumptions requisite for use of the general linear model. Individual embryos were thus grouped into tertiles of ECN (1–5 cells/6–7 cells/8–12 cells) and EFS (1/2/3–5), and using ordinal logistic regression modeling these were specified as a function of continuous metal concentrations, adjusting for age as a continuous variable [23], cigarette smoking as the dichotomous variable "never"/"ever" [24] and race/ethnicity as the dichotomous variable "non-Asian"/"Asian" [25]. 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 [26]. These models represent a variation on binary logistic regression, which more effectively captures information intrinsic to an ordered variable by assessing the effect for $k-1$, rather than for a single, outcome. Adjusted odds ratios (aOR) and 95% confidence intervals (CIs) were estimated by exponentiation of ordinal logistic regression model coefficients and associated confidence intervals. Covariates were selected for inclusion in ordinal logistic regression models following review of the literature and incorporation into directed acyclic graphs (DAGs) to identify confounders of potential associations between metals and embryo quality indicators. DAGs use causal graphing theory to identify a minimally sufficient set of variables with which to control confounding under a postulated causal pathway [27]. Embryo-level outcomes were anticipated to be correlated within couple violating the 'independence' assumption required for unbiased error estimates during ordinal logistic regression. Generalized estimating equations (GEE) were thus used to provide unbiased standard errors [28]. Cumulative logit plots corroborated the plausibility of the proportional odds assumption.

SAS v. 9.1.3 (SAS Institute, Cary, NC) was used for all statistical analyses. Statistical significance was defined as $P<0.05$ for a two-tailed test. Consistent with the hypothesis generating aim of this study, no adjustments were made to accommodate type-1 error inflation resulting from the conduct of multiple statistical tests.

## 3. Results

### 3.1. Demographic, clinical and exposure factors

Distributions for demographic and clinical factors describing female patients and their male partners are presented in Table 1. Among women, BMI is inversely correlated to EFS cohort score ($r=-0.34$, $P=0.016$). Few women (16.7%, $n=9$ at the time of specimen collection) and men (19.4%, $n=7$ at the time of specimen collection) report having ever smoked cigarettes. A substantial proportion of female (29.6%, $n=16$), but fewer male participants (14.7%, $n=5$) report Asian race/ethnicity. On average, Asian women (6.64 cells vs. 5.44 cells; $P=0.041$) and men (7.40 cells vs. 5.50 cells; $P=0.064$) generated embryos with greater ECN cohort score than did 'non-Asian'. There is a trend towards a positive association between TMC and ECN cohort score ($r=0.37$, $P=0.063$). ICSI was employed for most couples (63.6%), and resulted in a significantly ($P=0.044$) lower median EFS cohort score (2.00) than did conventional insemination (2.43). A median of 6 embryos (range 1–18) were generated per couple. As expected, embryos transferred on day 3 had a higher ECN cohort score than those transferred on day 2 (6.50 vs. 3.00; $P<0.0001$). The primary infertility diagnosis received was "Unexplained" (34.6%), followed by "Male factor" (21.8%). Approximately half of the women (50.9%) received a long luteal stimulation protocol.

Table 2 presents the distributions for metals measured in blood and urine specimens, which we were unable to normalize using data transformations. Median concentrations for blood Hg ($P=0.048$) and blood Pb ($P=0.001$) are lower for women (3.03 μg/L and 0.81 μg/dL, respectively) than for men (4.18 μg/L and 1.32 μg/dL, respectively). In contrast, median urine Cd concentrations are higher for women than men before creatinine-correction (0.34 and 0.16 μg/L, respectively; $P=0.004$) and following creatinine-correction (0.30 and 0.13 μg/g, respectively; $P<0.0001$). Despite these differences concentrations among couples are correlated between women and men for blood Hg ($r=0.57$, $P=0.001$), and demonstrate a trend for blood Pb ($r=0.48$, $P=0.085$), however, not for urine Cd prior to ($r=0.10$, $P=0.580$) or following creatinine-correction ($r=0.004$, $P=0.984$). Female age and blood Hg concentration demonstrate a tendency towards an inverse correlation ($r=-0.28$, $P=0.054$), whereas male age and blood Pb concentration trends towards a positive correlation ($r=0.46$, $P=0.071$). Mercury concentration in men is inversely associated with EFS cohort score ($r=-0.36$, $P=0.042$). Male blood Pb concentration demonstrates a trend towards a positive correlation with TMC ($r=0.57$, $P=0.089$). Median concentration for creatinine-corrected urine Cd is higher among Asian compared to non-Asian men (0.17 and 0.12 ng/g creatinine, respectively; $P=0.027$).

### 3.2. Multivariable analysis

We detect a 75% decrease in the adjusted odds for an increased ECN per μg/dL increase in blood Pb concentrations, measured in 24 women who generated 190 embryos (Table 3). This association is adjusted for blood Hg, urine Cd and creatinine, age, race/ethnicity,