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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Associations between IVF outcomes and essential trace elements measured in follicular fluid and urine: a pilot study

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Abstract
Purpose A hypothesis-generating pilot study exploring associations between essential trace elements measured in follicular fluid (FF) and urine and in vitro fertilization (IVF) endpoints.
Methods We recruited 58 women undergoing IVF between 2007 and 2008, and measured cobalt, chromium, copper, manganese, molybdenum, and zinc in FF ($n = 46$) and urine ($n = 45$) by inductively coupled plasma mass spectrometry (ICP-MS). We used multivariable regression models to assess the impact of FF and urine trace elements on IVF outcomes, adjusted for age, body mass index, race, and cigarette smoking.
Results Trace elements were mostly present at lower concentrations in FF than in urine. The average number of oocytes retrieved was positively associated with higher urine cobalt, chromium, copper, and molybdenum concentrations. FF chromium and manganese were negatively associated with the proportion of mature oocytes, yet urine manganese had a positive association. FF zinc was inversely associated with average oocyte fertilization. Urine trace elements were significant positive predictors for the total number of embryos generated. FF copper predicted lower embryo fragmentation while urine copper was associated with higher embryo cell number and urine manganese with higher embryo fragmentation. No associations were detected for implantation, pregnancy, or live birth.
Conclusions Our results suggest the importance of trace elements in both FF and urine for intermediate, although not necessarily clinical, IVF endpoints. The results differed using FF or urine biomarkers of exposure, which may have implications for the design of clinical and epidemiologic investigations. These initial findings will form the basis of a more definitive future study.

Capsule Our results suggest the importance of trace elements in both FF and urine for intermediate, although not necessarily clinical, IVF endpoints.

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Introduction

Over the last 10 years, in vitro fertilization (IVF) use has approximately tripled among U.S. women [1, 2]. IVF accounted for 99% of all assisted reproductive technologies used in 2013; 36% of cycles resulted in clinical pregnancies and 82% of those pregnancies resulted in a live birth [2]. In spite of live birth rates being similar to spontaneous conception, it is within the best interest of the clinical community to maximize live birth rates from IVF, given the attendant financial costs [3], psychological

stress [4], and health concerns [5–7]. To date, very few studies have assessed the impact of essential trace elements on IVF, although sufficient stores are clearly needed for success [8]. Cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), molybdenum (Mo), and zinc (Zn) are essential for normal physiologic function and play significant roles in human [9–12] and overall mammalian reproduction [13]. However, few human data are available to describe the role of essential trace elements in IVF, and their impact remains inconclusive.

The primary aim of this pilot study was to generate hypotheses describing the impact of follicular fluid (FF) Co, Cr, Cu, Mn, Mo, and Zn concentrations on IVF endpoints, to guide a more definitive future investigation. FF bathes a developing oocyte and so is a logical biomarker of the local, microfollicular environment, the quality of which may impact oocyte competence, embryo quality, implantation, and live birth [14, 15]. As a secondary aim, we explored differences between FF and urine trace elements to evaluate the validity of urine as an alternative biomarker for the FF microenvironment. In clinical laboratory medicine, trace element status is typically assessed through analysis of serum or plasma specimens. However, the analysis of urine for the essential trace elements is increasingly used in biomonitoring studies to assess population exposure to multiple trace elements considered toxic at higher levels. The results of this study will be used in the design of a more definitive future investigation of nutrition-based interventions to improve IVF outcomes.

Methods

Sample selection and clinical protocol

Participant recruitment and clinical protocols were previously described in detail [16, 17]. Briefly, between September 1, 2007 and August 31, 2008, 58 women completing a first IVF cycle at the University of California San Francisco (USCF) Center for Reproductive Health were enrolled in a prospective study to assess the impact of trace element exposures on IVF endpoints. The women underwent an initial infertility evaluation to confirm ovulation and uterine and tubal sufficiency; to maximize the number of participants, we enrolled women irrespective of the infertility diagnosis. Per clinic guidelines, the women underwent gonadotropin-induced ovarian stimulation according to a long-luteal down regulation protocol (long luteal and demi-halt), antagonist protocol (E_2-priming agonist and agonist), or flare protocol (microdose flare and mixed microdose flare). Human chorionic gonadotropin (hCG) was administered once follicles reached adequate size (≥17-mm diameter); oocytes were retrieved 36 h later. Mature oocytes in metaphase-2 (M2) arrest were fertilized by conventional insemination or by intracytoplasmic sperm injection (ICSI), using fresh semen collected on the day of retrieval or a frozen specimen from the partner or a donor. Fertilized zygotes were identified by the appearance of two pronuclei 16–18 h later. Embryo quality was assessed as embryo fragmentation score (EFS) and embryo cell number (ECN) 48 to 72 h after fertilization; higher EFS is generally associated with poorer IVF prognosis [18] and higher ECN is generally associated with a better IVF prognosis [19]. Embryos were transferred on day 2 or 3 post-fertilization based on clinical factors, and a serum hCG test was administered 14 days later to assess implantation. "Clinical pregnancy" was confirmed by the appearance of one or more gestational sacs on ultrasound 4 weeks after embryo transfer [20]. Nine months after transfer, patients' obstetricians were contacted to capture live birth outcomes.

Exposure assessment

Biospecimen collection was described previously [16, 17]. Briefly, FF was collected during oocyte retrieval by ultrasound-guided transvaginal fine needle aspiration. We pooled 0.5 to 5.0 mL FF from the single largest dominant follicle in each ovary and centrifuged for 10 min at 1500×*g* after removal of the oocyte for clinical use. FF is typically discarded as biologic waste. FF was available for 48 women, although we excluded $n = 2$ with evidence of blood contamination, leaving $n = 46$ for the current study. A fasting spot urine specimen was also collected from women ($n = 45$) on the day of oocyte retrieval. Biologic specimens were processed immediately and frozen at −80 °C until overnight shipment on dry ice to the Clinical Trace Elements section of the Laboratory of Inorganic and Nuclear Chemistry at the Wadsworth Center, New York State Department of Health (Albany, NY, USA).

Six trace elements (Co, Cr, Cu, Mn, Mo, and Zn) were determined in diluted FF using a Thermo Scientific Element 2 sector field-inductively coupled plasma mass spectrometer (SF-ICP-MS) (Bremen, Germany) [21]. This method validated for SF-ICP-MS offers greater sensitivity for small volumes of FF analyzed and resolves polyatomic interferences that are problematic for FF. Urine trace elements were determined using a method optimized for a Perkin Elmer ELAN DRC II single quadrupole ICP-MS instrument (Shelton, CT, USA) equipped with dynamic reaction cell technology (DRC-ICP-MS). This is a well-validated biomonitoring method for trace elements in urine that produces high quality data traceable to SI units. Method detection limits (MDL) for trace elements were calculated based on three times the standard deviation of the mean values of trace elements at low concentrations (i.e., matrix blank). We did not impute quantities below the MDL, but used the instrument generated data (including zeros and negative values) to avoid the introduction of bias that can occur with the former approach [22]. The laboratory staff was blinded to clinical data and to the study outcomes.

Statistical analysis

We characterized the distributions of trace elements measured in FF and urine, and demographic and clinical factors. We defined the proportion of mature oocytes as the number of oocytes retrieved in M2 arrest divided by the total number of oocytes retrieved from women undergoing ICSI. We defined the proportion of oocytes fertilized as the number of pronuclear zygotes divided by the total number of mature oocytes injected from women undergoing ICSI, or for women undergoing conventional insemination, divided by the number of oocytes with a visible polar body. We defined mean EFS as the average of fragmentation scores assigned to each woman's embryos and mean ECN as the average of cell numbers counted for each woman's embryos. We evaluated bivariate associations among FF trace elements, demographic factors, and clinical factors using Spearman correlation coefficients, Wilcoxon rank-sum tests, and Kruskal-Wallis tests as appropriate; we previously reported bivariate associations for urine trace elements [23]. To correct for difference in urine dilution among the women, all urine trace element were adjusted for creatinine [24] and are expressed as μg/g creatinine.

Using multivariable regression approaches, we explored associations between trace elements and IVF outcomes, adjusted for confounders selected a priori based on the literature, including age (years), BMI (kg/m^2), race ("other"/"Asian"), and current or past cigarette smoking ("never"/"ever") [25–28]. Trace element data were natural log-transformed after adding a constant to accommodate negative values and zeros, as were continuous study outcomes to satisfy distributional assumptions and to stabilize variances. To evaluate count endpoints, including total number of oocytes retrieved and total number of embryos generated per woman, we used negative binomial regression models. We used multiple linear regression models to evaluate continuous endpoints, including proportions of mature and fertilized oocytes per woman, average EFS and ECN per woman, and endometrial thickness (mm). We used modified Poisson regression, employing a sandwich variance estimator to accommodate correlated outcomes, to assess dichotomous oocyte or embryo-level endpoints [29, 30] including oocyte maturity (ICSI only cases), oocyte fertilization, and embryo quality indicators defined according to clinical experience as "bad" (≥3) vs. "good" (<3) EFS and "bad" (<6) vs. "good" (≥6) ECN. We exponentiated the regression coefficients and 95% confidence intervals (CI) from modified Poisson regression models to provide relative risk estimates (RR). We identified influential observations as FF and urine trace elements with DFBeta > |1|, which were omitted and the regression repeated [31]. All statistical tests were conducted using SAS v.9.4 (SAS Institute, Inc. Cary, NC, USA). We defined statistical significance as $P < 0.05$ for a two-tailed test.

Results

Demographic and clinical factors of the study sample

The median age of study participants was 36 years with a range of 28 to 43. Median BMI was 22.3 kg/m^2 with 75% below 25.3 kg/m^2. Few participants ($n = 6$, 13.0%) reported past or current cigarette smoking, whereas a considerable proportion (n=14, 30.4%) self-identified as Asian, and the remainder almost entirely white. Almost half of the women were diagnosed as female factor infertility ($n = 18$, 39.1%), $n = 12$ (26.1%) reported male partner infertility, while $n = 15$ (32.6%) diagnoses were unexplained. There were $n = 33$ (71.7%) ICSI cases.

Distributions of essential trace elements in follicular fluid

Distributions of FF trace elements are presented in Table 1. With the exception of Cr (69.6% < MDL), a majority of trace elements measured in FF were found to be above the MDLs. There were no statistically significant differences for FF trace element concentrations by infertility diagnosis (female factor vs. unexplained vs. male factor) or ovarian stimulation protocol (long-luteal protocols vs. antagonist protocols vs. flare protocols) (Supplemental Table S1). We detected weak to moderate positive correlations among FF trace elements (Supplemental Table S2), in which Co was correlated to Cr ($r = 0.32$; $P = 0.04$) and Mn ($r = 0.32$; $P = 0.03$), Cr was correlated to Mn ($r = 0.46$; $P = 0.002$), and Mn was correlated to Mo ($r = 0.32$, $P = 0.04$).

Essential trace elements in follicular fluid and IVF outcomes

Table 2 shows effect estimates and 95% CIs for regression models describing FF trace elements as predictors of woman-level IVF outcomes, adjusted for age, BMI, race, and cigarette smoking. We excluded $n = 2$ women missing covariate data, leaving $n = 44$ for the FF models. The proportion of oocytes in M2 arrest per woman was inversely associated with FF Cr levels (β = −0.51; 95% CI −0.82, −0.20; $P = 0.002$). Higher FF Zn was associated with a lower average proportion of oocytes fertilized per woman (β = −0.30; 95% CI −0.58, −0.02; $P = 0.04$), higher FF Co was associated with a lower average ECN per woman (β = −0.39; 95% CI −0.75, −0.04; $P = 0.03$), and higher FF Co was associated with a thicker endometrium (β = 0.20; 95% CI 0.03, 0.37; $P = 0.02$). No associations were detected for implantation, pregnancy, or for live birth.

Table 3 provides effect estimates and 95% CIs for regression models describing FF trace elements as predictors of oocyte-level and embryo-level IVF outcomes, adjusted for age, BMI, race, and cigarette smoking. We detected a lower likelihood for an oocyte to be recovered in M2 arrest with higher FF Cr (relative risk (RR) = 0.59; 95% CI 0.49, 0.73;

Table 1 Distribution of follicular fluid (FF) and urine essential trace elements in women

Trace elements	MDL	n < MDL	% < MDL	Min	P25	Med	P75	Max
FF, $n = 46$ women (μg/L)								
Cobalt*	0.05	2	4.3	<MDL	0.08	0.09	0.11	0.23
Chromium*	0.20	32	69.6	<MDL	<MDL	<MDL	0.37	3.56
Copper*	57.0	0	0	570	986	1124	1223	1732
Manganese*	0.10	0	0	0.15	0.29	0.38	0.65	1.97
Molybdenum*	0.60	2	4.3	<MDL	0.90	1.15	1.35	2.45
Zinc	21.0	0	0	<MDL	401	467	550	687
Urine, $n = 45$ women (μg/g creatinine)								
Cobalt	0.09	3	6.7	<MDL	0.22	0.27	0.35	0.79
Chromium	0.80	3	6.7	<MDL	0.96	1.78	2.73	6.01
Copper	1.70	6	13.3	<MDL	6	7.88	10.2	32.6
Manganese	0.60	11	24.4	<MDL	0.75	1.00	1.35	2.55
Molybdenum	1.00	0	0	9.95	29.3	36.04	48.04	79.2
Zinc	9.40	0	0	65.75	244	356.90	693.61	1752.83

Max maximum value, *Med* median value, *MDL* method detection limit, *Min* minimum value, *P25* twenty-fifth percentile, *P75* seventy-fifth percentile

* $P < 0.01$ for median difference between FF and urine ($n = 45$)

$P = 0.001$) and FF Mn (RR = 0.87; 95% CI 0.78, 0.97; $P = 0.01$) concentrations. However, higher levels of Cu in FF were associated with better embryo quality, assessed as EFS (RR = 2.50; 95% CI 1.16, 5.37; $P = 0.02$).

Distributions of essential trace elements in urine

Distributions of urine trace elements are also presented in Table 1. Overall, trace element concentrations were higher in urine than those in FF, perhaps due to the former comprising a major excretion route from the blood compartment, whereas the latter more likely comprises a plasma "ultrafiltrate." Median concentrations of urine Co ($P = 0.001$), Cr ($P = 0.003$), Mn ($P = 0.001$), and Mo ($P = 0.001$) were significantly higher than their respective FF measurements, although urine Cu concentration was significantly lower than that for FF ($P = 0.001$), and Zn concentrations were similar in urine and FF ($P = 0.91$). With the exception of urine having higher Mo concentrations among the women with unexplained infertility ($P = 0.05$), there were no statistically significant differences for urine trace element concentrations by infertility diagnosis or by ovarian stimulation protocol (Supplemental Table S1). We detected weak to moderate positive correlations between urine and FF trace elements (Supplemental Table S2). Urine Co was correlated with FF Co ($r = 0.40$; $P = 0.01$), urine Cu with FF Cu ($r = 0.35$, $P = 0.02$), urine Mo with FF Mo ($r = 0.56$, $P < 0.001$), and urine Zn with FF Zn ($r = 0.48$, $P = 0.001$). Furthermore, urine Co was correlated with FF Cr ($r = 0.48$; $P = 0.001$), urine Cu with FF Cr ($r = 0.38$; $P = 0.01$) and with FF Mn ($r = 0.33$, $P = 0.03$), and urine Mo with FF Cr ($r = 0.33$, $P = 0.03$).

Essential trace elements in urine and IVF outcomes

Table 2 also depicts effect estimates and 95% CIs for regression models describing urine trace elements as predictors of woman-level IVF outcomes, adjusted for age, BMI, race, and cigarette smoking. We excluded $n = 2$ women missing covariate data, leaving $n = 43$ for the urine models. The total number of oocytes retrieved on average per woman was associated with higher urine Co (β = 0.28; 95% CI 0.07, 0.49; $P = 0.01$), Cr (β = 0.49; 95% CI 0.19, 0.78; $P = 0.001$), Cu (β = 0.31; 95% CI 0.16, 0.47; $P = 0.0001$), Mn (β = 0.22; 95% CI 4.0×10^{-3}, 0.45; $P = 0.05$), and Mo (β = 0.31; 95% CI 0.11, 0.50; $P = 0.002$) levels. Similarly, the total number of embryos generated per woman was positively associated with all the measured urine elements: Co (β = 0.27; 95% CI 0.05, 0.48; $P = 0.01$), Cr (β = 0.47; 95% CI 0.15, 0.79; $P = 0.004$), Cu (β = 0.23; 95% CI 0.06, 0.39; $P = 0.01$), Mn (β = 0.34; 95% CI 0.09, 0.59; $P = 0.01$), Mo (β = 0.29; 95% CI 0.09, 0.49; $P = 0.01$), and Zn (β = 0.22; 95% CI 0.02, 0.42; $P = 0.03$). In contrast, no associations were detected for implantation, pregnancy, or for live birth.

Table 3 also depicts effect estimates and 95% CIs for regression models describing urine trace elements as predictors of oocyte-level and embryo-level IVF outcomes, adjusted for age, BMI, race, and cigarette smoking. In contrast to the FF results, higher urine Mn was associated with a higher likelihood for recovering an oocyte in M2 arrest (RR = 1.14; 95% CI 1.02, 1.28, $P = 0.02$), as well as with greater embryo fragmentation (RR = 0.81, 95% CI 0.67, 0.98; $P = 0.03$). Similar to FF, a higher urine Cu concentration was associated with better embryo quality, assessed as ECN (RR = 1.46; 95% CI 1.13, 1.88; $P = 0.003$). We did not

Table 2 Effect estimates (95% CI) for follicular fluid (FF) and urine essential trace elements with IVF outcomes among women

IVF endpoints	Essential trace elements					
	Co	Cr	Cu	Mn	Mo	Zn
FF ($n = 44$)						
Total oocytes[a]	−0.14 (−0.60, 0.32)	−0.10 (−0.53, 0.33)	0.75 (−0.10, 1.60)	0.01 (−0.25, 0.026)	1.1×10^{-3} (−0.49, 0.48)	0.11 (−0.69, 0.91)
Proportion M2-oocytes[b, c, d]	−0.34 (−0.70, 0.20)	**−0.51 (−0.82, −0.20)***	0.18 (−0.57, 0.92)	−0.14 (−0.30, 0.02)	0.04 (−0.25, 0.34)	−0.37 (−0.88, 0.15)
Proportion oocytes fertilized[b, c]	0.04 (−0.15, 0.24)	−0.09 (−0.25, 0.06)	−0.04 (−0.36, 0.29)	−0.04 (−0.13, 0.06)	0.03 (−0.14, 0.21)	**−0.30 (−0.58, −0.02)***
Total embryos[a, e]	−0.02 (−0.54, 0.51)	−0.11 (−0.57, 0.35)	0.50 (−0.35, 1.35)	−0.06 (−0.33, 0.20)	0.21 (−0.27, 0.69)	−0.11 (−0.94, 0.71)
Mean EFS[b, c, f]	−0.18 (−0.58, 0.22)	−0.15 (−0.44, 0.15)	−0.42 (−1.00, 0.15)	-4.0×10^{-3} (−0.19, 0.18)	1.0×10^{-3} (−0.33, 0.34)	−0.36 (−0.92, 0.20)
Mean ECN[b, c, e]	**−0.39 (−0.75, −0.04)***	0.16 (−0.16, 0.48)	0.04 (−0.58, 0.65)	−0.03 (−0.21, 0.15)	0.27 (−0.06, 0.60)	−0.22 (−0.81, 0.36)
Endometrial thickness[b, c, f]	**0.20 (0.03, 0.37)***	0.02 (−0.14, 0.18)	0.18 (−0.17, 0.52)	0.02 (−0.08, 0.12)	−0.06 (−0.24, 0.13)	0.08 (−0.23, 0.38)
Implantation[e, g]	1.40 (0.55, 3.59)	0.93 (0.30, 2.89)	0.47 (0.16, 1.41)	0.75 (0.42,1.37)	1.08 (0.39, 3.00)	0.43 (0.10, 1.85)
Pregnancy[e, g]	1.42 (0.51, 3.95)	1.19 (0.39, 3.60)	0.42 (0.12, 1.46)	0.73 (0.36, 1.48)	1.13 (0.32, 4.02)	0.51 (0.09, 2.78)
Live birth[e, g]	1.59 (0.47, 5.35)	1.63 (0.53, 5.06)	0.32 (0.07, 1.47)	0.90 (0.41, 1.96)	1.71 (0.52, 5.58)	0.75 (0.12, 4.63)
Urine[h] ($n = 43$)						
Total oocytes[a, b]	**0.28 (0.07, 0.49)***	**0.49 (0.19, 0.78)***	**0.31 (0.16, 0.47)***	**0.22 (4.0×10^{-3}, 0.45)***	**0.31 (0.11, 0.50)***	0.19 (−0.01, 0.40)
Proportion M2-oocytes[b, c, d]	−0.02 (−0.24, 0.20)	0.14 (−0.08, 0.35)	−0.06 (−0.22, 0.09)	0.10 (−0.11, 0.30)	0.07 (−0.16, 0.30)	0.01 (−0.32, 0.58)
Proportion oocytes fertilized[b, c]	0.03 (−0.09, 0.14)	0.10 (−0.02, 0.22)	−0.04 (−0.13, 0.04)	1.0×10^{-3} (−0.12, 0.12)	−0.02 (−0.14, 0.11)	0.01 (−0.08, 0.09)
Total embryos[a, e]	**0.27 (0.05, 0.48)***	**0.47 (0.15, 0.79)***	**0.23 (0.06, 0.39)***	**0.34 (0.09, 0.59)***	**0.29 (0.09, 0.49)***	**0.22 (0.02, 0.42)***
Mean EFS[b, c, f]	0.05 (−0.16, 0.25)	0.07 (−0.19, 0.33)	−0.02 (−0.17, 0.14)	0.02 (−0.21, 0.25)	1.02×10^{-3} (−0.23, 0.23)	−0.03 (−0.19, 0.13)
Mean ECN[b, c, e]	0.15 (−0.05, 0.36)	0.20 (−0.05, 0.45)	**0.15 (1.7×10^{-3}, 0.30)***	0.05 (−0.19, 0.28)	0.17 (−0.06, 0.40)	0.08 (−0.08, 0.24)
Endometrial thickness[b, c, f]	0.07 (−0.05, 0.18)	0.06 (−0.07, 0.19)	3.0×10^{-3} (−0.09, 0.09)	−0.02 (−0.15, 0.12)	1.11×10^{-3} (−0.13, 0.13)	0.01 (−0.08, 0.10)
Implantation[e, g]	0.88 (0.56, 1.37)	0.64 (0.35, 1.19)	0.90 (0.62, 1.29)	0.17 (0.59, 2.29)	0.05 (0.68, 1.61)	0.78 (0.52, 1.18)
Pregnancy[e, g]	0.76 (0.45, 1.49)	0.89 (0.44, 1.82)	0.81 (0.54, 1.21)	1.16 (0.50, 2.71)	0.90 (0.53, 1.53)	0.85 (0.51, 1.41)
Live birth[e, g]	0.66 (0.37, 1.19)	0.85 (0.36, 2.36)	0.71 (0.45, 1.12)	1.03 (0.40, 2.67)	0.80 (0.40, 1.59)	0.69 (0.35, 1.32)

NOTE: Statistically significant effects ($P < 0.05$) in boldface type

ECN embryo cell number, *EFS* embryo fragmentation score, *M2* metaphase-2 arrest

[a] Negative binomial regression coefficients with natural log-transformed essential elements as predictors and adjusted for age, BMI, race, and cigarette smoking

[b] Natural log-transformed value

[c] Linear regression coefficients with natural log-transformed essential elements as predictors and adjusted for age, BMI, race, and cigarette smoking

[d] Intracytoplasmic sperm injection (ICSI) only ($n = 33$)

[e] $n = 2$ missing values

[f] $n = 3$ missing values

[g] Modified Poisson regression relative risks with natural log-transformed essential elements as predictors and adjusted for age, race, and cigarette smoking

[h] Additional adjustment for urine creatinine (mg/dL)

* $P < 0.05$

Table 3 Relative risks (95% CI) for follicular fluid (FF) and urine essential trace elements among oocytes ($n = 475$) and embryos ($n = 294$)

IVF outcomes[a]	Essential trace elements[a]					
	Co	Cr	Cu	Mn	Mo	Zn
FF						
M2-oocyte[b]	0.77 (0.56, 1.06)	**0.59 (0.49, 0.73)***	1.08 (0.68, 1.72)	**0.87 (0.78, 0.97)***	1.08 (0.81, 1.44)	0.78 (0.52, 1.16)
Fertilized oocyte	1.03 (0.62, 1.73)	0.85 (0.69, 1.05)	1.01 (0.69, 1.46)	0.87 (0.70, 1.07)	1.12 (0.76, 1.64)	0.64 (0.38, 1.09)
Good EFS[c]	0.96 (0.60, 1.56)	0.97 (0.64, 1.47)	**2.50 (1.16, 5.37)***	0.93 (0.73, 1.18)	0.80 (0.60, 1.07)	1.25 (0.66, 2.37)
Good ECN[d]	0.93 (0.54, 1.59)	1.09 (0.72, 1.63)	1.17 (0.69, 1.99)	1.10 (0.87, 1.40)	1.39 (0.79, 2.43)	1.27 (0.60, 2.69)
Urine[e]						
M2-oocyte[b]	1.08 (0.93, 1.26)	1.00 (0.88, 1.13)	0.96 (0.88, 1.05)	**1.14 (1.02, 1.28)***	1.09 (0.95, 1.25)	1.01 (0.92, 1.10)
Fertilized oocyte	1.12 (0.93, 1.36)	1.04 (0.86, 1.27)	0.91 (0.79, 1.06)	1.06 (0.82, 1.37)	0.97 (0.81, 1.16)	1.03 (0.90, 1.18)
Good EFS[c]	0.92 (0.67, 1.28)	0.99 (0.77, 1.27)	1.04 (0.85, 1.28)	**0.81 (0.67, 0.98)***	0.91 (0.63, 1.29)	1.09 (0.91, 1.31)
Good ECN[d]	1.30 (0.97, 1.73)	1.26 (0.99, 1.59)	**1.46 (1.13, 1.88)***	1.19 (0.88, 1.59)	1.24 (0.95, 1.63)	1.13 (0.95, 1.34)

NOTE: Statistically significant effects ($P < 0.05$) in boldface type

ECN embryo cell number, *EFS* embryo fragmentation score, *M2* metaphase-2 arrest

[a] Modified Poisson regression relative risks with natural log-transformed essential elements as predictors and adjusted for age, race, and cigarette smoking

[b] ICSI only ($n = 396$)

[c] Mean EFS dichotomized as ≥3 = 0 ("bad") and <3 = 1 ("good")

[d] Mean ECN dichotomized as ≥6 = 1 ("good") and <6 = 0 ("bad")

[e] Additional adjustment for creatinine

* $P < 0.05$

detect associations for urine Co, Cr, Mo, or Zn with oocyte- or embryo-level outcomes.

Discussion

The results of this hypothesis-generating study suggest that essential trace element levels impact IVF endpoints. We identified unexpected negative associations between FF Cr and the proportion of mature oocytes among those retrieved and FF Zn with the proportion of oocyte fertilized. Furthermore, higher FF Co levels were associated with a lower average ECN per women, despite a positive association with endometrial thickness. Yet, the results differed using urine biomarkers as, with one exception, higher concentrations of essential trace elements were consistently associated with positive IVF outcomes. Higher urine Co, Cr, Cu, Mn, and Mo were associated with more oocytes retrieved per woman, and all the urine elements were associated with more embryos generated per woman. Curiously, higher FF Mn predicted a lower likelihood for retrieval of an oocyte in M2 arrest, whereas higher urine Mn predicted a higher likelihood. However, we did not detect significant associations for clinical endpoints, including implantation, pregnancy, and live birth. Our findings suggest that Co, Cr, Cu, Mn, Mo, and Zn affect intermediate IVF endpoints; however, the importance of levels measured in the follicle at the time of oocyte retrieval is unclear.

Trace element concentrations measured in this study mostly fell within the testing laboratory's upper reference interval for spot urine specimens. The laboratory-developed upper reference interval for trace elements in spot urine specimens was established based on guidelines provided by the Clinical Laboratory Standards Institute [32], and is defined as the 97.5th percentile based on data from a population study of adult men and women ($n = 1876$): Co (<1.74 μg/L), Cr (<2.12 μg/L), Cu (<35.7 μg/L), Mn (<2.20 μg/L), Mo (<233 μg/L), and Zn (<1808 μg/L). Although we lacked a strict comparison to other infertile women, urine trace elements generally aligned with levels for similarly aged U.S. women [33, 34]. All the study subjects had urinary trace element values that were below the laboratory-established upper reference limit, with the exceptions of Cr and Mn, possibly indicating differences between the population studied here and other biomonitoring projects completed in the same laboratory using the same analytical methodology. We did not find compelling evidence to suggest that controlled ovarian stimulation or infertility diagnosis had meaningful impacts on FF or urine essential trace element concentrations.

Previous research suggested the importance of blood Zn levels among women for spontaneous conception [35], yet the role for IVF is unclear. In vitro animal models suggest that insufficient Zn concentrations prevent M2 arrest, thereby decreasing fertilization rates [36]. The importance of Zn in FF may also derive from its biological role in moderating

oxidative stress via mediation of reactive oxygen species (ROS). Zn-supplemented media during in vitro maturation (IVM) of porcine blastocysts significantly decreased ROS levels in mature oocytes, likely in association with increased superoxide dismutase (SOD) activities and higher intracellular glutathione levels [37]. Similarly, Zn media supplementation (3.6–19 μmol) was associated with better quality mouse embryos [38, 39], yet higher concentrations (25 μmol) were embryotoxic [39]. Still, an earlier study of 33 human follicles found no association between Zn and oocyte fertilization during IVF [40]. The mean FF Zn (720 μg/L) in that study was higher than the maximum value measured in this study (687 μg/L). Higher blood Zn was associated with lower fecundability in a prospective study of women trying to conceive, though of borderline statistical significance [35]. However, a study of IVF patients reported higher FF Zn levels in pregnant women with endometriosis than those in non-pregnant women with endometriosis [41], although the means were similar to ours (478 μg/L), with values of approximately 475 and 525 μg/L for women with endometriosis and with tubal infertility, respectively. Given the role of Zn in suppressing oxidative stress, the inverse association between FF Zn and oocyte fertilization in our study was unexpected, although the positive association for total embryos generated and urine Zn was consistent. It is tempting to speculate that excessive FF Zn may suppress ROS requisite for cell viability, although further research is needed to clarify this result.

There are few available data to characterize the impacts of Mo, Mn, Cr, Cu, and Co on IVF. Low-dose Mo supplementation (5 mg/L) to female mice was associated with higher quality M2 arrest phase oocytes, as well as with lower ovarian oxidative stress [42]. However, Mo doses greater than 40 mg/L were associated with a decrease in blastocyst quality and lower ECN in preimplantation mice embryos [42]. A study of bovine oocytes reported inverse relationships between Mn concentrations in IVM medium and cumulus cell DNA damage, accompanied by positive associations with blastocyst development and embryo cell number, suggesting that Mn facilitates embryo development and decreases ROS [43]. The results were consistent with induction of Mn-based SOD activity, also associated with reduced FF ROS, and greater oocyte competence [14, 15, 41, 44]. Similarly, higher FF Cu-Zn SOD is associated with an optimized follicular environment via reduced ROS [45]; a study of rat dams fed a Cu-deficient diet (<0.5 μg Cu/g) had decreased embryo quality compared to those fed a Cu-sufficient diet (8.0 μg Cu/g) [46]. However, a Japanese study of 41 oocytes reported no associations for 40 essential elements measured in human FF and fertilization or embryo quality [47]. Co is an integral component of vitamin B_{12} (i.e., cobalamin), and higher FF or serum levels have been associated with improved embryo quality and with a higher proportion of live births from IVF [48, 49]. We identified no previous studies to characterize the impact of FF Cr on IVF outcomes, further underscoring the need for additional research into the role of essential FF trace elements in IVF.

In this study, we compared our results using FF and urine as biomarkers for essential trace elements. Curiously, we detected more associations using urine than FF, and with greater consistency to our a priori expectations. Higher levels of Co, Cr, Cu, Mn, Mo, and Zn in urine were associated with better IVF outcomes when we used women, oocytes, and embryos as units of observation. Some results appeared contradictory, such as associations between higher urine Mn and higher proportions of oocytes retrieved in M2 arrest, yet also with higher embryo fragmentation score. Apparent contradictions may reflect a chance finding, given our small sample size, or alternately indicate varying effects of essential trace elements at different stages of oocyte and embryo development. Based on our results, urine may not provide a comparable surrogate for the follicular microenvironment at the time of oocyte retrieval; however, it may provide a more informative biomarker of essential trace elements clearance and their impact on IVF endpoints, possibly due the low levels and limited variances for most FF trace elements measured.

Though novel, our study results are preliminary, having been limited by several important factors, and should be interpreted with caution. First, the small sample size and consequently low statistical power led to imprecise effect estimates and is likely to have diminished our ability to detect modest associations. This might explain in part the null results for implantation, pregnancy, and live birth. Further, this may account for unexpected observations, possibly reflecting chance. Second, we collected FF from only a single follicle, and so previously reported follicle-to-follicle variability within woman may bias some of our results towards the null hypothesis [17]. Additionally, we did not adjust for follicle diameter, which may impact FF trace element concentrations [50]. Still, we analyzed FF from only the largest dominant follicle, at least 17 mm in diameter, and so resulting exposure misclassification is likely to have been minimal. Third, we used spot urine collections, which were recently reported to be poor surrogates for characterizing variability and long-term exposure to some trace elements, including Co, Cu, and Mo [51], perhaps having led to exposure misclassification with bias towards the null, further undermining statistical power. Still, gold-standard 24-h urines are inconvenient, and there is greater chance for exogenous contamination as participants open, close, and transport the collection container. Fourth, while urine is typically used in biomonitoring studies to assess human exposure to essential and nonessential trace elements, clinical laboratory protocols recommend analyzing blood serum or plasma to assess trace element status, including deficiency as well as overload conditions, so measuring urine trace elements may not accurately reflect exposure. Fifth, it is likely that interactions exist among the essential trace elements we measured in FF and urine, or furthermore with

clinical factors, such as infertility diagnosis, ovarian stimulation, and ICSI; however, the limited number of participants prevented our investigating these possibilities, and so a larger sample is required for further consideration. Finally, reproduction is a couple-level process and our analysis incorporated the female partner only. Prior research describes associations between essential trace elements in seminal plasma and semen quality parameters among infertile couples, and so is also likely to impact IVF outcomes [52]. A more complete future assessment will require capture of male partners as well, ideally with incorporation of trace elements levels from both partners in joint statistical models predictive of IVF outcomes.

To the best of our knowledge, this is the first investigation to compare essential trace element levels measured in human FF and urine, and only the second study to assess the impact of FF essential trace elements on IVF endpoints. The different relationships detected between trace elements and IVF outcomes using FF and urine biomarkers suggested that urine may be better suited for epidemiologic and clinical studies; this is an unexpected result but also re-assuring given the highly invasive nature of FF retrieval. Still, we were unable to detect associations for implantation, pregnancy, and live birth, which are the clinical endpoints of greatest concern. Women undergoing IVF comprise a highly select group whose health-related behaviors and dietary habits might lead to different trace element exposure patterns compared to women with unrecognized or untreated infertility, or to women conceiving spontaneously [1, 53]. Thus, making generalizability of our results questionable. However, participation in our study was unlikely to be related to FF or urine trace elements, concentrations of which were presumably unknown by the participants, helping to ensure the validity of our results [54]. These preliminary findings warrant a larger study to evaluate the impact of Co, Cr, Cu, Mn, Mo, and Zn on IVF outcomes more definitively for possible future integration into diagnostic staging and treatment protocols.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interest.

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