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Paternal Exposure to Non-essential Heavy Metal Affects Embryo Cleavage and Implantation in Intracytoplasmic Sperm Injection (ICSI) Cycles: Evidence for a Paradoxical Effect

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Abstract

Although the adverse effects of non-essential heavy metals on semen quality have been demonstrated in experimental animal models and occupational human exposure studies, little is known about the reproductive efficiency of exposed sperm during the process of intracytoplasmic sperm injection (ICSI). Our study aims to evaluate the effect of paternal exposure to non-essential heavy metals on embryo efficiency outcomes (embryo cleavage, fragmentation, implantation, and live birth) in ICSI cycle. Ninety-five heterosexual couples who underwent 95 ICSI cycles and 78 fresh embryo transfers between January 2003 and December 2009 were evaluated**.** Men whose female partner was undergoing ICSI were asked to provide semen and blood samples. Heavy metal levels (Pb, Cd, As, Hg, Ba, and U) were analyzed using an ion-coupled plasma-mass spectrometry (ICP-MS; Agilent 7500 ce, Agilent Technologies, Germany) equipped with a cell dynamic range (CDR). Paternal exposure to trace heavy metals was found to influence intermediate reproductive endpoints in ICSI cycles. After adjusting for paternal and maternal confounders, paternal blood concentrations of Cd [−0.30(−0.11,−0.02)], As [−0.26(−0.16,−0.11)], and U [−0.22(−0.24,−0.02)] were inversely associated with embryo cell cleavage on day 3. Counterintuitively, paternal blood and semen Pb levels [0.26(0.01,0.22); 0.25(0.03,0.14)] as well as semen U levels [0.27(0.01,0.19)] were positively associated with the proportion of implanted embryos. There were no significant associations observed for clinical pregnancy and live birth rates with any paternal heavy metal concentrations in semen and blood. These findings highlight the importance of paternal health for embryo efficiency outcomes in ICSI treatment cycles and the need for more male partner inclusive counseling in fertility practice. They also underline a paradoxical positive association between some heavy metal pollutants at low exposure levels and reproductive outcomes.

Keywords Intracytoplasmic sperm injection · Embryo cleavage · Implantation · Live birth · Heavy metals

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Introduction

As a result of the industrial revolution, an exponential increase in environmental pollutants was observed in numerous countries across the world [1, 2]. Heavy metals, such as lead (Pb), cadmium (Cd), arsenic (As), barium (Ba), mercury (Hg), and uranium (U), are non-essential xenobiotics that carry substantial implications on reproductive health in humans [2–4]. Because of their widespread cumulative burden, there is growing concern for reproductive adverse effects even with low-level exposures.

The adverse effects of high-level exposure for non-essential heavy metals on male and female reproductive health have been clearly demonstrated in experimental animal models and

in occupational human exposure studies [5–8]. The clinical impact of low-level and nonoccupational exposure on human reproduction, however, is less conclusive [9]. Available studies suffer several shortcomings and are often limited by their retrospective design, low number of observations, and lack of adjustment to relevant confounders [9, 10]. In a previous prospective cohort study, we demonstrated significant inverse associations between sperm viability and semen Pb, Cd, Ba, and U levels in men presenting to an infertility clinic [10]. We also showed associations between semen U concentrations and increased odds for below-reference progressive sperm motility and morphology.

Ingested in trace amounts over a prolonged time duration, non-essential heavy metals are believed to increase oxidative damage to cells either by de novo generation of reactive oxygen species or by depletion of available antioxidant enzymes [11]. Divalent metals may also bind to the estrogen receptor potentially interfering with intracellular sex-steroid hormone signaling pathways [12].

Although the adverse effects of low-level exposure to environmental pollutants on reproductive endpoints remain less well-defined, some evidence suggests that exposed infertile women undergoing assisted reproduction may be particularly at risk [13–17]. Paternal contribution to reproductive toxicity is now thought to be more important than previously thought. Experimental animal studies have demonstrated positive associations between preconception male parent comorbidities and adverse fetal development and poor birth outcomes [18–20]. In humans, there is scarcity of data evaluating the effects of paternal comorbidities on obstetrical and birth outcomes. Paternal smoking for instance was associated with a higher body mass index in male newborns, while paternal diabetes was linked to preterm birth [21, 22]. More recently, a retrospective study of healthcare claims reconfirmed the presence of significant associations between paternal comorbidities and preterm birth, low birth weight, and neonatal intensive care unit stay [23].

Very few studies have evaluated the effects of paternal exposure to environmental pollutants on reproductive endpoints during intracytoplasmic sperm injection (ICSI) treatment cycles. Intuitively, sperm cells share their genome with the embryo, in addition to organelles and elements, which are essential for oocyte activation, fertilization, embryo cleavage, and implantation. On the other hand, there is growing evidence in favor of transgenerational effects of environmental pollutants suggesting a possible impact for preconception parental exposure on specific embryonal and fetal events [24]. By increasing oxidative stress, non-essential heavy metals may alter epigenetic programming through binding to acetyl groups interfering with gene expression [25]. By modifying sex-steroid hormone signaling pathways, heavy metals may further influence epigenetic patterning [26]. Pb, Cd, Hg, and U, for instance, when bound to estrogenic receptors, appear to mediate a stimulatory effect, whereas As exerts an inhibitory one [12]. Epigenetic processes in the male germline are believed to be different from somatic cells. During mammalian development, global epigenetic reprogramming occurs shortly after fertilization as sperm DNA gets deprived from its parental methylation signature [27]. Modified reprogramming of epigenetic patterns occurring in coincidence may be transmitted and maintained through the germline for future generations, potentially influencing embryonal behavior and fetal development [27].

While preconception health remains an essential component of fertility counseling, the association between paternal exposure to pollutants and reproductive endpoints in intracytoplasmic sperm injection cycles remains uncertain and poorly explored. The primary aim of this study was therefore to address this existing data gap and generate hypotheses by identifying associations for further confirmation between paternal levels of non-essential heavy metals (Pb, Cd, As, Hg, Ba, and U) in blood and semen and reproductive endpoints (embryo cleavage, fragmentation, implantation, and live birth). To accomplish this aim, we investigated heterosexual couples undergoing ICSI treatment and participating in the Environment and Male Infertility (EMI) study, a prospective cohort investigation of environmental determinants of male infertility. Generated data are expected to guide future clinical research.

Material and Methods

Study Participants

This is a prospective cohort study of heterosexual couples undergoing ICSI treatment using autologous oocytes at the Haifa Idriss Fertility Center of the American University of Beirut Medical Center, Beirut, Lebanon. It is part of a wider research initiative, the Environment and Male Infertility (EMI) study, which explores environmental determinants of male infertility [10].

All women participants ($n = 95$) were 18 to 40 years of age, had a BMI of 18 to 30 Kg/m^2, had completed one fresh IVF/ICSI cycle between January 2003 and December 2009, and had their male partner provide blood and semen samples for the measurement of non-essential heavy metal levels (Pb, Cd, As, Hg, Ba, and U) within 1 month from cycle initiation. At recruitment, participants completed a research questionnaire regarding demographic characteristics, medical history, occupational and lifestyle exposures, etiology and duration of infertility, and past fertility treatments. The following clinical entities were defined as follows: diminished ovarian reserve, when baseline FSH was ≥12 mIU/mL and/or estradiol ≥50 pg/mL; recurrent pregnancy loss (RPL), as three or more consecutive clinical miscarriages; and recurrent implantation failure (RIF), as three or more failed IVF/ICSI cycles. It should be

noted that the human personnel, culture media source, and laboratory techniques were unchanged throughout the study period.

The study was approved by the Human Studies Institutional Review Board of the American University of Beirut, and all eligible participants signed an informed consent form prior to enrollment.

Study Protocols and Outcome Measures

All women were evaluated with baseline pelvic ultrasound, FSH, and estradiol serum levels prior to initiation of treatment. All participants were treated as per clinic protocol with human menopausal gonadotropins (HMG) for ovarian stimulation using a luteal gonadotropin-releasing hormone (GnRH) agonist protocol for pituitary suppression. Gonadotropin dose selection was done based on age, BMI, ovarian reserve testing, and past ovarian response. Dose adjustments were made according to the ultrasound follicular response. Human chorionic gonadotropins (hCG) were used to trigger final follicle maturation when at least three dominant follicles ≥17 mm were observed.

All collected oocytes were fertilized by ICSI using sperm from the male partner. A two-step discontinuous density gradient process was used for sperm preparation. Evaluation of oocyte maturation, pronuclear development, and embryo scoring was done as per laboratory protocol. Oocytes were classified as germinal vesicle, metaphase I, or metaphase II. Fertilization rate was assessed 18–20 hours following insemination and defined as the number of zygotes with two pronuclei divided by the number of MII oocytes inseminated. Cleavage rate was defined as the number of embryos with ≥6 blastomeres on day 3 of development divided by the number of zygotes. All embryo transfers (n = 78) were performed on day 3 of cleavage. Cleavage stage embryos were assigned a grade as follows: grade A, blastomeres of equal size with ≤20% cytoplasmic fragmentation; grade B, blastomeres of equal or unequal size, with cytoplasmic fragmentation ranging from 20–50%; and grade C, blastomeres of any size with >50% cytoplasmic fragmentation. The overall embryo quality score (EQS) was categorized as EQS-I when ≥2/3 of transferred embryos were grade A; EQS-II when <2/3 were grades A and C; and EQS-III when ≥2/3 were grade C.

Clinical outcomes were evaluated per embryo transfer. A clinical pregnancy was defined as the presence of an intrauterine pregnancy confirmed by ultrasound and a live birth as the delivery of a live neonate at ≥24 weeks gestation. Implantation rate was defined as the number of intrauterine gestational sacs detected on ultrasound divided by the number of embryos transferred.

Specimen Collection and Heavy Metal Analyses

Men whose female partner was undergoing ICSI were asked to provide a semen sample following an abstinence period of 3–7 days. Semen was collected into a sterile wide-mouth metal-free polypropylene container by masturbation while wearing metal-free gloves. All metal components in the semen collection room were carefully covered with plastic material.

Seminal fluid aliquots (0.5 mL) were digested with 70% nitric acid (HNO3) (Fisher Scientific, USA), filtered, and diluted to 5 mL. Digestion was performed in a closed vessel microwave system (temperature program reaching 120°C and sustained for 10 min at 1000 W). Deionized water from Milli-Q system was used for dilution before performing the analyses. All glassware were washed and immersed in 5% HNO3 overnight and then rinsed with deionized water before use.

Heavy metal levels (Pb, Cd, As, Hg, Ba, and U) in seminal fluid were analyzed using an ion-coupled plasma-mass spectrometry (ICP-MS; Agilent 7500 ce, Agilent Technologies, Germany) equipped with a cell dynamic range (CDR) as previously described [10]. For quality control, certified reference materials were used as per manufacturer, and measurements of analytes found to be within standard range. Quantification of analytes was done by the standard addition of 0, 1, 5, and 10 ppb of heavy metals. Responses were measured for the series of the standards added, and results were plotted and then extrapolated to y=0 to get the value of the metal. To assess the instrument performance, the standard reference material was analyzed after each 20 samples. One blank tube containing deionized water was added to each batch of samples. Metal levels in all blank samples were lower than the limits of detection (LOD). The LOD for Hg was 0.5 and 1 μg/L for all other metals. For statistical purposes, no censoring of concentrations below the detection limit was implemented [28]. Machine read values were analyzed as described and were reported in μg/L.

Statistical Analysis

Descriptive statistics [mean, median, standard deviation (SD) and standard error of the mean (SE), proportion (%)] were used to describe the population demographic characteristics, distributions of heavy metals in the seminal fluid and blood, and reproductive outcome measures (Table 1). Normal distribution was evaluated using Kolmogorov-Smirnov analysis. Categorical variables are presented as frequencies and proportions. The ANOVA analysis was performed for parametric variables, the Mann-Whitney U nonparametric tests for nonparametric variables, and the chi-square test for categorical variables.

Associations between selected variables and outcome measures were assessed using different univariate and multivariate

Table 1 Demographic and reproductive characteristics of women participating in the EMI Study, grouped by pregnancy success

Characteristics	All cycles *n*=95	Pregnant *n*=40	Nonpregnant *n*=38	*P* value*
Age, years ± SD	33.4 ± 6.0	33.4 ± 6.1	33.7 ± 5.9	*NS*
Duration of infertility, years ± SD	5.29 ± 3.1	4.8 ± 2.9	5.7 ± 3.2	*NS*
Primary etiology of infertility, *n* (%)				*NS*
Male factor	45 (57.7)	24 (60.0)	21 (55.3)	
Female factor	22 (28.2)	11 (50.0)	11 (50.0)	
Unexplained	11 (14.1)	5 (12.5)	6 (15.8)	
Diminished ovarian reserve[a], *n* (%)	8 (10.3)	3 (7.5)	5 (13.2)	*NS*
Recurrent implantation failureb , *n* (%)	25 (32.1)	11 (27.5)	14 (36.8)	*NS*
Recurrent pregnancy lossc, *n* (%)	13 (16.9)	6 (15.0)	7 (18.9)	*NS*
Women smoking history				
Ever smoker, *n* (%)	11 (10.9)	5 (12.5)	6 (15.8)	*NS*
Men smoking history				
Ever smoker, *n* (%)	48 (47.5)	20 (50.0)	17 (44.7)	*NS*
Cigarettes per week, *n* ± SD	41.3 ± 85.2	25.4 ± 55.1	25.4 ± 56.9	*NS*
Men alcohol consumption history				
Ever drinker, n (%)	47 (46.5)	21 (52.5)	17 (44.7)	*NS*
Consumption per week, *n* ± SD	2.7 ± 7.4	1.8 ± 4.8	3.6 ± 10.2	*NS*
Semen parameters				
Concentration, *n* ± SD	34.7 ± 35.7	36.7 ± 32.3	36.4 ± 40.8	*NS*
Mobility, %	46.6	45.5	47.3	*NS*
Morphology, %	40.3	38.9	43.1	*NS*
Mode of fertilization				
Intracytoplasmic sperm injection, *n* (%)		40 (100)	38 (100)	*NS*
Metaphase II oocytes retrieved, *n* ± SD	8.7 ± 4.7	9.3 ± 4.4	8.1 ± 5.0	*NS*
Cleavage rate, %	54.0	58.6	50.8	*NS*
Embryos available for transfer, *n* ± SD	4.6 ± 3.5	5.1 ± 2.9	4.3 ± 3.9	*NS*
Day of embryo transfer				
D3, n (%)	78 (100)[d]	40 (100)	38 (100)	
Embryos transferred, median ± SE	3 ± 0.2	4 ± 0.3	3 ± 0.4	*0.04*
Embryo Quality score, median ± SE	1 ± 0.1	1 ± 0.1	2 ± 0.1	*0.03*
Implantation rate, %	22.5			
Miscarriage rate, *n* (%)	4 (10.0)			
Clinical pregnancy per transfer, *n* (%)	40 (51.3)			
Live birth per transfer, *n* (%)	36 (46.2)			

SD, standard deviation; SE, standard error

Diminished ovarian reserve was defined as [basal FSH ≥ 12 mIU/mL and/or basal E2 ≥ 50 pg/mL]

[b] Defined as ≥ 3 past failed attempts

[c] Defined as ≥ 3 consecutive pregnancy loss

[d] Seventeen cycles (17.9%) did not reach the stage of embryo transfer because of suboptimal performance in one of the following indicators: follicle response, oocyte maturation, or embryo cleavage

*Significance levels (*P* value < 0.05) when comparing pregnant versus nonpregnant study groups.

regression models. Potential confounders were assessed on the basis of statistical and biological considerations. A significance of 0.05 was set for inclusion of variables into the multivariate model, whereas 0.1 was the cutoff for exclusion. Explored confounders included men and women age, men and women current smoking history and alcohol consumption history, number of previous IVF/ICSI failures, embryo quality score (EQS), and number of embryos transferred. Some parameters were retained in the multivariable models as covariates because of biological relevance despite lack of statistical significance.

Metal measurement data were natural log-transformed after adding a constant to accommodate negative and null values, to satisfy distributional assumptions, and to stabilize variances. Multiple linear regression models were used to detect linear trends and evaluate continuous endpoints, including proportions of cleaved embryos with ≥6 blastomeres on day 3, embryo quality/fragmentation scores, and implanted embryos per woman. We used modified Poisson regression, employing a sandwich variance estimator to accommodate correlated outcomes, to assess the occurrence of reproductive events: clinical pregnancies and live births.

Metal concentrations in the semen and blood of male partners were also categorized into quartiles to allow for nonlinear effects while investigating dose-dependent relationships, with the lowest quartile considered as the reference group. A multivariable logistic regression model was used to assess the correlations between single metal categories and live birth as a dichotomous outcome. Tests of trends in ordinal metal categories were performed using regression models with integer values.

All statistical analyses were conducted using SPSS version 25 (IBM Corporation, USA). For all analyses, a P value < 0.05 was regarded as statistically significant for two-tailed significance tests.

Table 2 Comparison of heavy metal concentrations in the semen and blood of men participating in the EMI Study, stratified by ICSI reproductive outcome

Variable	Pregnant (n = 40)	Nonpregnant (n = 38)	P value*
Seminal fluid			
Pb	5.40 (21.67 ± 7.63)	4.33 (9.01 ± 1.87)	*NS*
Cd	4.61 (31.93 ± 11.20)	4.08 (59.18 ± 46.20)	*NS*
As	17.95 (27.89 ± 3.65)	17.00 (41.51 ± 10.12)	*NS*
Ba	10.20 (56.71 ± 26.26)	8.75 (82.83 ± 41.98)	*NS*
Hg	20.10 (64.73 ± 21.22)	12.45 (62.03 ± 35.28)	*NS*
U	1.60 (4.12 ± 1.70)	1.15 (2.54 ± 0.69)	*NS*
Blood			
Pb	33.93 (46.19 ± 6.49)	29.44 (36.14 ± 3.90)	*NS*
Cd	5.21 (14.77 ± 4.54)	6.67 (20.30 ± 9.11)	*NS*
As	12.69 (20.42 ± 5.88)	13.18 (19.52 ± 2.63)	*NS*
Ba	9.5 (188.12 ± 86.45)	7.10 (76.89 ± 29.30)	*NS*
Hg	12.80 (21.48 ± 4.21)	12.65 (70.63 ± 46.01)	*NS*
U	0.55 (1.42 ± 0.55)	0.64 (1.14 ± 0.34)	*NS*

Values in μg/L are median (mean ± SE). * Significance levels (***P*** value < 0.05) when comparing pregnant versus nonpregnant study groups, using Mann-Whitney U nonparametric test

Results

A total of 95 heterosexual couples underwent 95 ICSI cycles with 78 fresh embryo transfers (Table 1). The mean age of women participants was 33.4 years (SD: ± 6; range: 22 to 40) and the mean duration of infertility was 5.29 years (SD: 3.1 years). Male factor was the primary cause of infertility in 57.7% of cycles. A history of recurrent implantation failure and recurrent pregnancy loss was observed in 32.1 and 16.9% of cycles, respectively. The luteal GnRH agonist pituitary suppression protocol was utilized in all cycles and all transfers were performed on day 3 of embryo development. Seventeen cycles (17.9%) did not reach the stage of embryo transfer because of suboptimal performance in one of the following outcomes: follicle response, oocyte maturation, or embryo cleavage. The cleavage rate was 54.0% and the implantation rate was 22.5%. A total of 244 embryos were replaced to the uterus resulting in 55 implanted gestational sacs observed on ultrasound. Clinical pregnancy and live birth rates per transfer were 51.3 and 46.2%, respectively.

The median concentrations of heavy metals in paternal semen were 5.4 μg/L for Pb, 4.18 μg/L for Cd, 17.60 μg/L for As, 10 μg/L for Ba, 13.2 for Hg, and 1.5 μg/L for U. The median distributions in paternal blood were 31.38 μg/L for Pb, 6.63 μg/L for Cd, 12.10 μg/L for As, 9.00 μg/L for Ba, 12.30 μg/L for Hg, and 0.60 μg/L for U. Table 2 presents the distributions for metals measured in paternal semen and blood stratified by pregnancy outcome. There were no significant differences in semen and blood concentrations for any of the heavy metals (Pb, Cd, As, Hg, Ba, and U) in the pregnant and nonpregnant groups.

Covariates Affecting Reproductive Outcomes

Given univariate models to identify covariates of statistically significant confounding effects on reproductive endpoints, woman age and smoking history were found to be positively associated with embryo cleavage rates (β = 0.31; 95% CI: 0.001, 0.028; P = 0.01, and β = 0.24; 95% CI: 0.01, 0.38; P = 0.04), respectively. Cigarette consumption in the male partner was negatively associated with cleavage rate (β = −0.25; 95% CI: −0.002, −0.000; P = 0.03). A history of alcohol drinking in the male partner was associated with a higher proportion of fragmentation in embryos on day 3 of transfer (β = −0.30; 95% CI: −0.68, −0.82; P = 0.01). Each additional failed IVF attempt was marginally associated with a lower likelihood of embryo implantation (β = −0.22; 95% CI: −0.051, 0.002; P = 0.07) and was negatively associated with clinical pregnancy and live birth rates (OR = 0.77; 95% CI 0.60, 0.99, and OR = 0.74; 95% CI 0.56, 0.98), respectively. A lower proportion of good quality embryos was inversely associated with clinical pregnancy and live birth (OR = 0.44; 95% CI 0.19, 1.00) (OR = 0.34; 95% CI 0.13, 0.83), respectively. Each additional embryo replaced on day 3 was

marginally associated with improved odds of clinical pregnancy (OR = 1.26; 95% CI 0.96, 1.65).

Heavy Metals Affecting Reproductive Outcomes

We analyzed the associations between paternal concentrations of heavy metals and embryo-level outcomes. Table 3 shows effect estimates and 95% CIs for linear regression models describing heavy metals in semen and blood, natural log-transformed, as predictors of embryo efficiency endpoints, adjusted for men and women age, men and women current smoking, and alcohol consumption history. There were no significant associations detected for the proportion of cleaved embryos, embryo quality/fragmentation scores (EQS), and implanted embryos with any of the heavy metal concentrations in semen. However, high blood Cd, As, and U levels in men were associated with significantly lower proportions of cleaved embryos ($\beta = -0.30$; 95% CI: −0.11, −0.02; $P = 0.01$) ($\beta = -0.26$; 95% CI: −0.16, −0.11; $P = 0.02$) ($\beta = -0.22$; 95% CI: −0.24, −0.02; $P = 0.05$), respectively. Unexpectedly, paternal blood and semen Pb concentrations were positively associated with embryo implantation ($\beta = 0.26$; 95% CI: 0.01, 0.22; $P = 0.03$) ($\beta = 0.25$; 95% CI: 0.03, 0.14; $P = 0.04$), respectively. Semen U concentrations were also positively associated with embryo implantation ($\beta = 0.27$; 95% CI: 0.01, 0.19; $P = 0.03$).

We also analyzed the associations between paternal concentrations of heavy metals and reproductive outcomes. Table 3 describes relative risk estimates and 95% CIs for modified Poisson regression models evaluating natural log-transformed metal levels as predictors of ICSI reproductive endpoints, adjusted for men and women age, men and women current smoking history, alcohol consumption history, number of previous IVF/ICSI failures, embryo quality score (EQS), and number of embryos transferred. There were no significant associations observed for clinical pregnancy and live birth rates with any paternal heavy metal concentrations in semen and blood.

Tables 4 and 5 show the odds ratio estimates and 95% CIs for multivariate logistic models assessing correlations between paternal metal concentration quartiles and live birth, adjusted for men and women age, men and women current smoking history, alcohol consumption history, number of previous IVF/ICSI failures, embryo quality score (EQS), and number of embryos transferred. Defined categories of heavy metal concentrations in the semen and blood of men participants did not predict the likelihood of live birth in ICSI treatment cycles.

Discussion

In this prospective cohort study of heterosexual couples undergoing ICSI treatment, we examined associations between paternal semen and blood levels of non-essential heavy metals and reproductive outcomes. In adjusted models, we detected associations between paternal blood trace elements and intermediate ICSI endpoints. Lower embryo cleavage rates were

Table 3 Effect estimates (95% CI) for heavy metals concentrations in the semen and blood of male partners with ICSI outcomes

IVF outcomes	Heavy metals					
Seminal fluid	Pb	Cd	As	Ba	Hg	U
Cleaved oocyte[a]	-0.03(-0.07,0.05)	0.03(-0.04,0.05)	0.02(-0.08,0.08)	0.03(-0.04,0.05)	-0.03(-0.06,0.05)	-0.15(-0.15,0.02)
Embryo Q-score[a]	0.12(-0.6,0.23)	0.11(-0.05,0.16)	0.17(-0.06,0.31)	0.03(-0.09,0.13)	0.08(-0.09,0.15)	0.03(-0.16,0.25)
Implantation[a]	**0.25(0.03,0.14)**	0.03(-0.04,0.06)	0.07(-0.06,0.12)	0.04(-0.04,0.06)	0.10(-0.04,0.08)	**0.27(0.01,0.19)**
Pregnancy[b]	1.1(0.85,1.44)	1.02(0.83,1.26)	1.00(0.67,1.49)	1.03(0.82,1.27)	1.05(0.83,1.32)	1.08(0.75,1.56)
Live birth[b]	1.13(0.86,1.50)	1.02(0.82,1.27)	1.03(0.68,1.56)	1.07(0.86,1.33)	1.06(0.83,1.35)	1.11(0.76,1.63)
Blood	Pb	Cd	As	Ba	Hg	U
Cleaved oocyte[a]	-0.07(-0.13,0.07)	**-0.30(-0.11,-0.02)**	**-0.26(-0.16,-0.11)**	-0.21(-0.07,0.01)	-0.10(-0.08,0.04)	**-0.22(-0.24,-0.02)**
Embryo Q-score[a]	0.07(-0.17,0.30)	0.11(-0.05,0.17)	0.14(-0.06,0.28)	0.03(-0.06,0.11)	0.23(-0.01,0.23)	0.02(-0.18,0.34)
Implantation[a]	**0.26(0.01,0.22)**	0.17(-0.02,0.09)	0.02(-0.6,0.110	0.12(-0.02,0.06)	-0.03(-0.07,0.05)	0.17(-0.08,0.17)
Pregnancy[b]	1.14(0.67,1,96)	1.02(0.77,1.36)	1.12(0.70,1.77)	1.06(0.88,1.27)	1.04(0.77,1.41)	1.00(0.55,1.82)
Live birth[b]	1.03(0.58,1.84)	1.04(0.76,1.41)	1.11(0.68,1.81)	1.01(0.83,1.24)	1.05(0.76,1.44)	0.83(0.40,1.71)

[a] Linear regression coefficients with natural log-transformed heavy metals as predictors and adjusted for women age, men and women current smoking history, and men alcohol consumption history

[b] Modified Poisson regression relative risks with natural log-transformed heavy metals as predictors and adjusted for women age, men and women current smoking history, men alcohol consumption history, number of previous ICSI failures, embryo quality score (EQS), and number of embryos transferred

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

Table 4 Associations between live birth and quartiles of heavy metals concentrations in the semen of men whose female partners underwent ICSI treatment

IVF outcome	Heavy metals			
Live birth (*n*=36)	Heavy metal quartiles (range in ng/mL)	Adjusted[a] OR (95% CI)	Heavy metal quartiles (range in ng/mL)	Adjusted[a] OR (95% CI)
	Pb		Cd	
	1 (LOD-2.48)	1.00	1 (LOD-1.57)	1.00
	2 (2.49-5.40)	1.15 (0.34-3.89)	2 (1.58-5.01)	1.23 (0.35-4.31)
	3 (5.41-11.85)	1.50 (0.35-6.35)	3 (5.02-16.29)	1.00 (0.21-4.71)
	4 (≥ 11.86)	1.12 (0,28-4.50)	4 (≥ 16.30)	1.20 (0.33-4.36)
	P trend[b]	0.96	*P* trend	0.98
	As		Ba	
	1 (LOD-13.60)	1.00	1 (LOD-5.00)	1.00
	2 (13.61-17.95)	3.33 (0.78-14.16)	2 (5.01-10.10)	2.60 (0.65-10.38)
	3 (17.96-38.50)	3.00 (0.73-12.25)	3 (10.11-26.90)	2.08 (0.52-8.34)
	4 (≥ 38.51)	2.14 (0.43-10.74)	4 (≥ 26.91)	2.97 (0.70-12.62)
	P trend	0.35	*P* trend	0.44
	Hg		U	
	1 (LOD-5.25)	1.00	1 (LOD-0.50)	1.00
	2 (5.26-11.14)	2.53 (0.45-14.23)	2 (0.51-1.50)	2.72 (0.62-12.05)
	3 (11.15-37.46)	2.96 (0.55-16.00)	3 (1.51-3.20)	1.46 (0.33-6.48)
	4 (≥ 11.47)	1.43 (0.26-7.82)	4 (≥ 3.21)	1.14 (0.24-5.32)
	P trend	0.33	*P* trend	0.47

OR, odds ratio. [a] All models were adjusted for women age, men and women current smoking history, men alcohol consumption history, number of previous ICSI failures, embryo quality score (EQS), and number of embryos transferred. [b] The *P* trend value was calculated using the median of each quartile as a continuous variable

associated with higher paternal blood levels of Cd, As, and U. Unexpectedly, a higher chance of embryo implantation was found to be associated with higher paternal semen and blood concentrations of Pb, as well as with higher paternal semen U. We did not detect nonetheless any significant associations for clinical endpoints, including clinical pregnancy and live birth rates.

Negative Effects of Trace Heavy Metals on Reproductive Endpoints

In this study, we found lower embryo cleavage rates to be associated with higher paternal blood levels of Cd, As, and U. Our understanding of preconception paternal exposure to environmental pollutants on fertility outcomes with assisted reproduction remains nevertheless extremely limited. We identified very few prior studies in the literature which describe the effects of heavy metal exposure in the male partner on human embryo quality outcomes.

In a prospective cohort study, Bloom MS et al. described inverse adjusted associations between blood Pb (OR 0.58, 95% CI 0.37–0.91, $P = 0.018$) and Hg (OR 0.60, 95% CI 0.45–0.79, $P = 0.0004$) in the male partner and embryo cell cleavage [13]. Higher blood Pb levels in men were also associated with increased embryo fragmentation scores (OR 1.47, 95% CI 1.11–1.94, $P = 0.007$) and higher urine Cd concentrations with low oocyte fertilization (RR 0.19, 95% CI 0.03–1.95). In our study, we observed no significant effects for paternal Pb and Hg in blood and semen on embryo quality outcomes, namely cleavage and fragmentation. It should be noted that the median distributions for blood Pb and Hg in our male population (31.38 and 12.30 μg/L, respectively) were several folds higher than those reported for the study sample of Bloom et al. (1.32 μg/L; 95% CI 1.04–2.11 and 4.18 μg/L; 95% CI 3.11–5.12, respectively) and for the US population of men corresponding to the same study period (1.49 μg/dL; 95% CI 1.41–1.58 and 0.81 μg/L; 95% CI 0.72–0.94) [2]. Although these results suggest the importance of trace exposures to toxic heavy metals across a continuum of ICSI endpoints and suggest temporal variability for reprotoxic effects, we found no evidence for any such adverse effects at the pregnancy level.

Due to the scarcity of human data, it may be valuable to explore the reproductive toxicity of non-essential heavy metals in experimental animal studies. Pb exposure in rodents was associated with dose-dependent reductions in testicular alkaline phosphatase and sodium-potassium ATPase activities and also in the production of reactive oxygen radicals leading to increased lipid peroxidation, loss of plasma membrane fluidity, and decreased sperm motility [29, 30]. Cd exposure was also found to be toxic to spermatogenesis by competing with

Table 5 Associations between live birth and quartiles of heavy metals concentrations in the blood of men whose female partners underwent ICSI treatment

IVF outcome	Heavy metals			
Live birth (*n*=36)	Heavy metal quartiles (range in ng/mL)	Adjusted[a] OR (95% CI)	Heavy metal quartiles (range in ng/mL)	Adjusted[a] OR (95% CI)
	Pb		Cd	
	1 (LOD-21.99)	1.00	1 (LOD-1.19)	1.00
	2 (22.00-32.56)	0.74 (0.17-3.16)	2 (1.20-6.55)	0.86 (0.23-3.16)
	3 (32.57-53.57)	0.53 (0.10-2.65)	3 (6.56-18.26)	0.78 (0.21-2.84)
	4 (≥ 53.58)	1.08 (0.23-5.01)	4 (≥ 18.27)	1.05 (0.26-4.26)
	P trend[b]	0.71	*P* trend	0.97
	As		Ba	
	1 (LOD-7.80)	1.00	1 (LOD-4.30)	1.00
	2 (7.81-12.10)	0.54 (0.13-2.25)	2 (4.31-9.00)	0.80 (0.21-3.02)
	3 (12.11-24.40)	1.29 (0.34-4.82)	3 (9.01-67.37)	1.87 (0.44-7.85)
	4 (≥ 24.41)	1.12 (0.27-4.63)	4 (≥ 67.38)	0.67 (0.18-2.46)
	P trend	0.62	*P* trend	0.57
	Hg		U	
	1 (LOD-4.35)	1.00	1 (LOD-0.20)	1.00
	2 (4.36-11.05)	1.08 (0.20-5.83)	2 (0.21-0.61)	0.35 (0.07-1.80)
	3 (11.06-21.47)	1.20 (0.24-5.9)	3 (0.62-1.50)	0.54 (0.11-2.59)
	4 (≥ 21.48)	0.38 (0.07-2.08)	4 (≥ 1.51)	0.67 (0.14-3.17)
	P trend	0.52	*P* trend	0.79

OR, odds ratio. [a] All models were adjusted for women age, men and women current smoking history, men alcohol consumption history, number of previous ICSI failures, embryo quality score (EQS), and number of embryos transferred. [b] The *P* trend value was calculated using the median of each quartile as a continuous variable

calcium for calmodulin binding interfering with protein tyrosine phosphorylation [30]. Furthermore, Cd was shown to reduce the phosphorylation of axonemal proteins by increasing peroxidation of membrane lipids. A detrimental effect of Cd on sperm metabolism is believed to be mediated by inhibition of glycogen phosphorylase, glucose-6-phosphatase, Mg-dependent ATPase, and succinic acid dehydrogenase [31]. Depleted U in rodents was found to pass through the blood-testis barrier by modulating the gene expression of molecules involved in the regulation of tight junctions and to accumulate in reproductive organs, seminal fluid, and spermatozoa [32]. Uranium exposure was associated with endocrine-mediated effects in rats with reduced testicular testosterone production, histologic atrophy of convoluted tubules, and increase in sperm DNA damage [25, 32].

Although researches from our group and others have confirmed associations between heavy metal exposures and abnormal semen quality endpoints, little is known about the reproductive efficiency of exposed sperm during the process of intracytoplasmic sperm injection (ICSI) [10]. While the direct placement of sperm inside the ooplasm is expected to bypass the natural stages of egg-sperm interaction during conventional fertilization, it remains unclear how paternal exposure could potentially affect embryo developmental dynamics. In such cases, it is conceivable that reproductive toxicity from chronic male partner exposure could be mediated by the delivery of heavy metals into the oocyte by direct entry through binding to the penetrating sperm. Along this line, it has been hypothesized that sulfhydryl groups in human sperm provide binding sites for divalent metals, like Cd, Pb, and Hg [33]. Intracellular binding to specific receptors could modify regulatory kinase pathways with long-term implications on oocyte fertilization, embryo implantation, and future fetal development. Inactivation of intracellular antioxidant molecules following entry into an oocyte might also increase oxidative damage followed by failure of cell mitosis and disruption of embryo development [11]. Alternatively, metal-induced reactive oxygen radicals could alter epigenetic expression of the sperm haplosome as demonstrated in rats for Cd and Pb [34]. It has also been suggested that heavy metals like Hg may bind to microtubules interfering with cytoskeletal physiology, cell division, and embryo development [35]. Furthermore, Hg and Pb have the capacity to bind to the estrogen receptor which could potentially disrupt sex-steroid hormone signaling and alter embryo growth [12]. It should be mentioned that preconception parental exposures to non-essential heavy metals might have long-bearing effects on obstetrical and birth outcomes as well, with paternal exposures being more frequently associated than maternal ones as suggested by longitudinal clinical studies [36]. The reported discordance between effects

for partner's exposures underscores the importance of capturing preconception exposure at the male partner level which was demonstrated by our study.

Paradoxical Effects of Trace Heavy Metals on Reproductive Endpoints

Our findings suggest that higher levels of Pb and U in male partners are associated with better reproductive outcomes when embryos were considered units of observation. The positive associations between embryo implantation rates and paternal Pb and U exposures were unexpected and counterintuitive.

In a preliminary prospective cohort study, Bloom et al. described significant adjusted associations between paternal blood Hg levels and lower embryo fragmentation (OR 0.85, 95% CI 0.72–1.00, $P = 0.044$) [16]. The same group also found paternal blood Pb to be a positive predictor for clinical pregnancy with borderline significance (RR = 1.39, 95% CI 0.99–1.96, $P = 0.06$) [13]. In our study, we found no associations between paternal blood Pb and Hg and embryo quality outcomes, namely cleavage and fragmentation. It should be noted that a paradoxical association between nonmetal pollutants and reproductive outcomes has also been reported previously by Jarrell et al., who found a strongly positive correlation between embryo cleavage rates and hexachlorobenzene (HCB) levels in women undergoing IVF ($r = 7.10$) [13].

It is highly likely that trace heavy metals may gain access to the regulatory mechanisms of reproductive cells by mimicking the ionic and molecular characteristics of essential elements. Consequently, they may interfere with gene expression and intracellular communication altering protein synthesis [36]. Specifically, Pb, Cd, and Hg have been shown to express physiologic estrogenic effects and modulate progesterone synthesis [12, 37]. While disruptions to intracellular physiologic events with reduced fecundity are often expected in association with high-level metal exposure, paradoxical surges in fertility have been described in men with low-level exposures [13, 37–39]. Variability in the direction of effects may therefore indicate a shift in susceptibility to trace heavy metals concurrent to changes in critical windows of vulnerability [13]. In our study, we found a paradoxical direction of effects for paternal U levels on embryo quality and embryo efficiency outcomes. While high paternal blood U levels were associated with decreased embryo cell cleavage, high semen U concentrations were associated with increased embryo implantation. One possible explanation is that trace heavy metals, by modulating apoptotic pathways, may contribute to the elimination of embryos of lower implantation potential, which could lead to enhanced implantation as a result of an improvement in the processes of natural selection [40].

It is now clear that trace heavy metals are associated with different pattern effects on various reproductive endpoints, suggesting a pathophysiologic pathway unique to each element. This is best exemplified by the reported effect pattern of heavy metals on obstetrical endpoints. In their prospective cohort study, Bloom et al. demonstrated that while higher paternal urine U levels were associated with lower birth weights in newborns, paternal urine As concentrations were positively linked to higher birth weights in the same study population [36]. A positive association was also detected for gestational age at delivery and birth length with paternal blood Hg [36]. These observations provide further evidence for the far-reaching effects of preconception paternal exposures to trace heavy metals onto the realm of obstetrical and neonatal outcomes.

Equally counterintuitive was the presence of a positive association between woman smoking and embryo cleavage ($\beta = 0.24$; 95% CI: 0.01, 0.38; $P = 0.04$). This is in line with data from a previous prospective cohort study, in which Hughes EG et al. demonstrated higher fertilization rates in women heavy smokers undergoing IVF with compared with non-smokers [41]. Similarly, Zenzes and Reed found that women smokers had a higher proportion of good quality embryos [40].

Although these findings may indicate varying effects of essential trace elements at various exposure levels and at different stages of embryo development, they may alternatively reflect chance occurrence given the small number of observations, the multiple comparisons, and the adjustment for multiple confounders; and consequently, we remain very cautious in making conclusive statements in that regard. This should not exclude nonetheless the need for a more detailed analysis of the effects of trace heavy metals contaminants on reproductive outcomes in couples undergoing assisted reproduction.

Variations in the Reproductive Effects of Trace Heavy Metals

We identified sparse studies characterizing the impact of paternal trace heavy metals on the outcomes of assisted reproductive technologies, which further underscores the need for additional research into the role of non-essential heavy metals in assisted reproduction. Inconsistencies in findings between studies about the reproductive toxicity of trace elements may be due to several factors, including differences in study design and in population characteristics.

Some of the conflicting results of human studies may conceivably be due to the assumption that not all individuals have similar predispositions to the reproductive toxic effects of trace heavy metals [10]. It is possible that damage to gametes is determined by alterations in the efficiency of the protective mechanisms operational at any specified target in any given individual. Such differential susceptibility may be cell- and organ-specific, but also individual-dependent. Individuals may therefore be expected to respond differently to the same toxic stimulus. Despite evidence on mechanisms mediating metal-induced

reproductive toxicity, less information is available on pathways which determine the susceptibility of male reproductive organs to toxic injury. These pathways may involve the expression of metal transporter mechanisms and specific regulatory proteins [42–44]. A nonlinear dose-response relationship might then be anticipated between non-essential heavy metals and reproductive endpoints, as protective regulatory mechanisms become increasingly overwhelmed by cumulative exposure. This phenomenon might be best exemplified by the a nonlinear inverted U-shaped dose-response curve seen among girls of Bangladeshi mothers with higher urine Cd levels in relation to fetal size [45].

Polymorphisms and genotype differences may represent yet another complex facet to the differential response of various heavy metals to the same endpoints. Polymorphisms have been identified in genes coding for thiol-containing metal-binding proteins, known as metallothioneins (MTs), which protect cells from metal toxicity [46–48]. Several isoforms of MTs have been acknowledged and found to be cell-type specific. The differential tissue expression of these metal carriers could therefore be responsible for organ and individual vulnerability to the toxic effect of these elements [46–48]. In a study from Korea, for instance, maternal blood Hg were associated with lower birth weights only in mothers with GST1 null genotype [49].

Strengths and Limitations

The major strengths of this study are (a) its prospective design which minimizes the risk of reverse causation; (b) the comprehensive adjustment of all meaningful male and female confounding variables more realistically representing the couple-driven nature of reproduction; and (c) the measurement of non-essential heavy metals in two body compartments as surrogate estimates of potential spermatogenic injury in men.

We emphasize nonetheless that the findings of this study are preliminary and should be interpreted with caution, because of the presence of some limitations. First, the limited number of observations leading to low statistical power is likely to yield inaccurate effect estimates and diminish the detection of modest associations. The number of covariates incorporated in regression models could have resulted in sparse strata and imprecise estimates, namely for interactions in which we detected differences of questionable clinical relevance. A larger sample size may be required to account for these effects. Second, the study design previewed a single spot blood and semen collection per participant. Spot measurements of elements may fail to account for semen sample variations, variable time exposures, and elimination half-lives of heavy metals, often leading to exposure misclassification and attenuation of risk estimates. Third, the absence of speciated measurements of arsenic does not take into account the differential toxicity of organic and inorganic species, which may also lead to exposure misclassification. Finally, reproductive outcome is dependent on a complex multifactorial process which involves both partners. Although confounders from both partners were accounted for in the final prediction models, measurements of metals incorporated the male partner only. It should be noted that we are currently conducting a prospective cohort research looking at environmental pollutant exposures from both partners in joint predictive models of ICSI outcomes.

These findings highlight the importance of paternal health for embryo efficiency outcomes in ICSI treatment cycles. While this study was limited to chronic low-level exposures, it is highly likely that as paternal exposure to trace heavy metals increases, the collateral reproductive effects become more relevant. Whereas counseling of women is common practice in fertility care, little has been achieved in that regard for the male partner. There are also scarce data available to characterize the impact of trace heavy metals on the outcome of ICSI, further underscoring the need for additional research into the role of non-essential trace elements in assisted reproduction.

Conclusions

To the best of our knowledge, this is one of very few reports to describe preconception paternal element exposures and reproductive endpoints with ICSI cycles. Most consistently, we detected negative associations between embryo cleavage rates and paternal blood Cd, As, and U levels. Unexpectedly, we also found positive associations between embryo implantation rates and paternal blood and semen Pb, as well as semen U levels. Although limited by its sample size, this study is unique in exploring prospective effects of paternal trace exposures on reproductive outcomes in ICSI cycles. The unexpected observation of positive associations between embryo implantation and paternal Pb and U exposures merits further investigation. We are currently exploring the impact of multiple environmental pollutants, including non-essential heavy metals and organic compounds, in both male and female partners on reproductive outcomes of ICSI cycles.

Although no associations were found for live birth following ICSI treatment and paternal heavy metal elements in blood and semen, obstetrical risks and long-term postnatal effects cannot be excluded. Longitudinal postnatal research on the well-being of children in relation to preconception parental exposure to heavy metals is therefore required.

Author's Contribution C.S., G.Z., A.G., J.A.: conception and design of study. C.S., N.A., N.H., D.F.: acquisition of data and approval of the final version. J.A., A.G.: analysis of data. J.A., C.S.: drafting of the manuscript. C.S., G.Z., A.G., J.A.: revising the manuscript critically for intellectual content.

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Availability of data and material Not Applicable

Code availability Not Applicable

Declarations

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the American University of Beirut.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Patients signed informed consent regarding publishing their data.

Conflicts of Interest/Competing Interests On behalf of all authors, the corresponding author states there is no conflict of interest.

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