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ASSISTED REPRODUCTION TECHNOLOGIES

Urine cortisol concentration as a biomarker of stress is unrelated to IVF outcomes in women and men

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Abstract
Purpose Our primary objective was to assess associations between urine cortisol as a biomarker of psychological stress and *in vitro* fertilization (IVF) outcomes. A secondary objective was to assess associations between toxic metals and cortisol.
Methods Urine and blood specimens were collected from 52 women and 28 male partners completing a first IVF procedure, on the day of oocyte retrieval. Urine cortisol was measured with an enzyme-linked immunosorbent assay. Mercury (Hg), cadmium (Cd), and lead (Pb) were determined in blood and Cd in urine by inductively coupled plasma-mass spectrometry.
Results No associations were indicated for cortisol with IVF outcomes in multivariable regression models adjusted for covariates. However, we detected positive linear associations for cortisol and urine Cd (β=9.96, 95%CI 1.52, 21.44) and blood Hg (β=1.44, 95%CI 0.31, 3.18). An exploratory stratified analysis suggested a potential inverse association between urine cortisol and oocyte fertilization among women with low, but not high blood Hg.
Conclusion While limited, these preliminary data suggest that psychological stress may not play a major role in IVF outcomes, which therefore could be one less concern for couples and their clinicians. Our data also raise the possibility for toxic metals to modify associations between cortisol and IVF outcomes among women. However, these preliminary results require corroboration in an experimental animal model and confirmation in a larger, more definitive observational study.

Keywords Cadmium (Cd) · Cortisol · *In vitro* fertilization (IVF) · Lead (Pb) · Mercury (Hg) · Stress

Capsule Psychological stress does not appear to impact *in vitro* fertilization outcomes, but there is a possibility for modification by exposure to toxic metals.

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Introduction

Fertility is sensitive to effects of psychological stress. Inverse associations have been reported between pregnancy and psychological stress among populations conceiving without medical assistance [1, 2]. Similarly, couples undergoing *in vitro* fertilization (IVF) are under substantial psychological stress, which has been hypothesized as a risk factor for treatment failure [3]. The time of oocyte retrieval and the time before embryo transfer are particularly stressful periods for IVF patients, raising concern as to the influence of stress on fertility outcomes [4, 5]. Thus, questions to be addressed

herein are related to the role of stress, using urine cortisol as a biomarker, on IVF outcomes.

Additionally, there has been increasing concern with regard to the effect of exposure to toxic agents, including metals, and possible detrimental effects on reproductive outcomes [6]. We previously reported associations between exposure to toxic metals and IVF outcomes [7–9] and other work reports changes in cortisol levels in association with Hg exposure [10] and Cd treatment [11, 12]. Given these associations, we also conducted a hypothesis-generating exploratory analysis to assess potential moderating effects for metals on associations between cortisol and IVF outcomes.

We know of only two prior studies that have considered urine cortisol as a biomarker for stress as it relates to IVF outcomes [13, 14], and to the best of our knowledge there exist no studies that have assessed the impact of toxic metals on associations between psychological stress and IVF outcomes. To address these data gaps, our primary aim was to further clarify associations between psychological stress, using urine as a biomarker, and IVF outcomes. As a secondary, hypothesis generating aim, we explored the impact of toxic metals on these associations.

Methods

Study sample and treatment protocol

The Study of Metals and Assisted Reproductive Technologies (SMART) consisted of 58 women and 36 men completing a first IVF cycle at the University of California at San Francisco (USCF) between March 2007 and April 2008. Detailed clinical, specimen collection, and participant selection protocols for the SMART were previously described in detail [9]. In brief, women underwent gonadotropin-induced ovarian stimulation to facilitate follicular maturation and endometrial development, monitored by serum estradiol levels and ultrasonagraphy. Upon maturation of a sufficient number of follicles (≥17 mm diameter), human chorionic gonadotropin (hCG) was administered to precipitate ovulation and oocytes were retrieved by fine needle aspiration 36 h later. All procedures were conducted between 9 AM and 1 PM.

We collected fasting whole blood and urine specimens from women and non-fasting whole blood and urine specimens from male partners, on the day of oocyte retrieval. These were immediately processed, transferred into 1.8 mL cryovials, and frozen at−80 °C. Specimens were shipped on dry ice to the Laboratory of Inorganic and Nuclear Chemistry at the Wadsworth Center, New York State Department of Health (Albany, NY) in May, 2008 for measurement of toxic metals. Residual urine specimens were transferred to the Frye Lab at the University at Albany (NY) in July, 2011 for cortisol analysis using a commercial enzyme-linked immunosorbent kit-based assay specific for measurement of urinary cortisol (Oxford Biomedical Research. Oxford, MI). The current study comprised 52 women and 28 male partners for which cortisol levels were available.

IVF outcomes included: number of oocytes retrieved, oocyte maturity (defined as an oocyte retrieved in metaphase-2-arrest among $n=33$ using intracytoplasmic sperm injection (ICSI)), normal oocyte fertilization (defined by the presence of two pronuclei 16–18 h post-insemination), number of embryos, mean embryo cell number (ECN), mean embryo fragmentation score (EFS), implantation, and pregnancy [7–9]. ECN was defined as the number of blastomeres counted for each embryo at the time of transfer, and is positively correlated to embryo quality. EFS was defined by an ordinal grade describing the extent of cytoplasmic fragmentation and is inversely correlated to embryo quality. A blood hCG test was performed 2 weeks after embryo transfer followed by a confirmatory test to determine implantation. Pregnancy was defined as visualization of a gestational sac by ultrasound two weeks later.

Laboratory analysis

Toxic metals including Hg, Cd, and Pb in blood and Cd in urine were determined by dynamic reaction cell inductively coupled plasma-mass spectrometry. Urine creatinine was measured using a commercially available colorimetric assay (Oxford Biomedical Research, Oxford, MI), as part of our earlier work. Detailed methods and quality control information are beyond the scope of the current report, but are described in detail elsewhere [9, 15]. Urine cortisol was analyzed using a commercially available enzyme-linked immunosorbent assay kit. A standard curve was established using eight concentrations run in duplicate (ranging from 0.0 to 10.0 ng/mL). Coefficients of variation derived from the standard concentrations averaged 10.6 %, with a minimum of 0.6 at 2 ng/mL and a maximum of 22.3 at 0.2 ng/mL. Single determinations were made for each sample and analyzed together on a single plate.

Statistical analyses

We evaluated distributions for demographic and clinical variables, cortisol, and toxic metals. We tested creatinine-adjusted bivariate associations between urinary cortisol levels and IVF outcomes as well as for sex, age, body mass index (BMI), calculated as weight divided by the square of height (for women), and toxic metals. We used linear regression to assess effects of cortisol on mean ECN and mean EFS, adjusted for age [16], cigarette smoking [17], and urine creatinine [15]. Poisson regression, employing a sandwich estimator of the variance [18], was utilized to examine associations for cortisol with oocyte maturity, oocyte fertilization,

implantation, and pregnancy, adjusting for covariates. Negative binomial models were employed for number of oocytes retrieved and number of embryos. Statistical significance was defined as $P<0.05$ for main effects using 2-tailed tests.

To explore associations between toxic metals and cortisol we used linear regression models with toxic metals as continuous predictors and urine cortisol as the dependent variable, assessing the impact of each metal adjusted for other metals as well as for covariates. To evaluate effect modification we conducted stratified regression analysis of urine cortisol and IVF outcomes by levels of significant toxic metal-cortisol predictors above and below the medians [19]. Potentially important associations were identified in stratified models using a false discovery rate (FDR) of 0.10. The FDR procedure accommodates type-1 error inflation from multiple statistical tests [20].

For all regression models we excluded influential observations with Dfbeta ≥ |1.96| and repeated the analysis [21]. We also adjusted for BMI in additional regression models [22]. We assessed *post hoc* statistical power for creatinine-adjusted differences in urine cortisol levels at $P<0.05$ between women with pregnancy and those without to further evaluate null results. SAS v.9.3 (SAS Institute Inc., Cary, NC) was employed for the analysis.

Results

On average, women were 35.7 and men 37.9 years of age. Asian race was reported by 26.9 and 14.3 % of women and men, respectively. Similar proportions of men (17.9 %) and women (15.4 %) reported a history of cigarette smoking. For primary infertility diagnosis, female factor was reported for 38.5 %, male factor for 23.1 %, with the unexplained and 'other' categories making up the remaining 38.5 % of couples. Mean (standard deviation (SD)) urine cortisol levels were similar between men and women (75.6 (46.1) and 74.8 (49.0) ng/g creatinine, respectively). An average of 13 oocytes was collected from each woman, 76.0 % of which were mature (ICSI only), and 66.0 % of which fertilized. An average of 7.3 embryos developed per couple, with overall mean ECN and EFS of 5.8 and 2.2, respectively. Half of procedures led to implantations and 43.8 % resulted in pregnancy. Median (interquartile range (IQR)) blood Hg and Cd, and urine Cd levels in μg/L, and blood Pb in μg/dL, were 2.96 (2.06), 0.27 (0.27), 0.35 (0.52), and 0.70 (0.49), respectively among women. Analogous values for men were 4.29 (3.21), 0.20 (0.16), 0.16 (0.18), and 1.40 (1.18), respectively.

No significant creatinine-adjusted bivariate correlations were detected between cortisol and IVF endpoints or with demographic variables, nor did we detect covariate-adjusted associations among women during regression modeling (Table 1). Results were similar for men (data not shown). In linear regression models adjusted for covariates and excluding $n=6$ influential observations, female urine Cd (β=9.96, 95%CI 1.52, 21.44) and blood Hg (β=1.44, 95%CI 0.31, 3.18) were significant predictors of urine cortisol. Adjustment for BMI did not meaningfully impact the results. Among men there was a tendency towards a positive association for urine Cd and cortisol (β=36.30, 95% CI−42.85, 115.45), although without significance.

In stratified models adjusted for covariates, only the effect of blood Hg on the association between cortisol and oocyte fertilization had on FDR <0.10 (Table 2). Whereas cortisol was inversely associated with fertilization among women with low blood Hg (i.e., below the median), no association was suggested for women with high blood Hg (i.e., above the median).

Discussion

In this pilot study of 52 women and 28 male partners, we did not detect associations between cortisol and IVF outcomes. Previous studies employing psychometric measures, biological stress measures, or both have reported varied, and often conflicting, results for associations between psychological stress and IVF outcomes [5, 13, 14, 23–26]. Infertility, as well as its treatment, can be considered significant chronic stressors, but the neuroendocrine components of this response and the extent to which environmental contaminants modulate this are unclear.

Table 1 Regression models of cortisol concentration on reproductive outcomes in women undergoing IVF

Outcome	β[1]	95%CI	*P*-value
#Oocytes retrieved[2]	0.01	−0.01, 0.02	0.53
Mature oocyte[3, 4]	0.01	−0.003, 0.02	0.15
Oocyte fertilized[4]	−0.001	−0.01, 0.01	0.95
Total embryos[2]	0.01	−0.01, 0.03	0.36
Mean ECN[5, 6]	0.08	−0.002, 0.15	0.06
Mean EFS[5]	−0.003	−0.03, 0.02	0.81
Implantation[4]	−0.01	−0.04, 0.02	0.50
Pregnancy[4]	−0.01	−0.040.02	0.48

CI, confidence interval; *ECN*, embryo cell number; *EFS*, embryo fragmentation score

Implantation confirmed by blood hCG tests. Pregnancy confirmed by ultrasound

[1] Adjusted for creatinine levels, age, and cigarette smoking

[2] Negative binomial regression model

[3] Among 33 cases of intracytoplasmic sperm injection

[4] Poisson regression model employing sandwich estimator of the variance

[5] Linear regression model

[6] Excluding $n=1$ influential observation

Table 2 Regression models of cortisol on reproductive outcomes in women undergoing IVF, stratified by median metal concentration

Outcome	n	β[1] (stratified by bld. Hg)	95 % CI	*P*-value	n	β[1] (stratified by urine Cd)	95 % CI	*P*-value
#Oocytes retrieved[2]								
<50 %	23	−0.01	−0.03, 0.02	0.66	26	0.01	−0.01, 0.03	0.43
>50 %	23	0.04	0.001, 0.08	0.05	26	−0.02	−0.07, 0.03	0.43
Mature oocyte[3, 4]								
<50 %	15	0.01	−0.02, 0.03	0.65	19	0.01	−0.01, 0.03	0.38
>50 %	13	0.004	−0.004, 0.01	0.35	14	0.04	0.01, 0.07	0.02
Oocyte fertilized[4]								
<50 %	23	−0.01	−0.02, −0.01	0.001*	26	−0.01	−0.02, 0.01	0.25
>50 %	23	0.01	−0.02, 0.03	0.68	26	0.05	−0.01, 0.11	0.12
Total embryos[2]								
<50 %	21	−0.01	−0.04, 0.01	0.28	25	0.004	−0.02, 0.03	0.74
>50 %	23	0.04	0.01, 0.07	0.01	25	0.03	−0.02, 0.08	0.25
Mean ECN[5]								
<50 %	21	−0.05	−0.14, 0.05	0.29	24	0.07[6]	−0.05, 0.19	0.22
>50 %	23	0.05	−0.06, 0.16	0.34	25	0.20	−0.03, 0.44	0.09
Mean EFS[5]								
<50 %	20	−0.02	−0.05, 0.02	0.36	25	−0.02	−0.05, 0.02	0.35
>50 %	23	0.02	−0.04, 0.08	0.56	24	0.03	−0.07, 0.13	0.53
Implantation[4]								
<50 %	20	−0.03	−0.07, 0.0004	0.05	25	−0.03	−0.07, 0.01	0.11
>50 %	22	−0.03	−0.11, 0.05	0.43	23	−0.02	−0.14, 0.11	0.78
Pregnancy[4]								
<50 %	20	−0.02	−0.06, 0.01	0.21	25	−0.03	−0.07, 0.01	0.11
>50 %	22	−0.04	−0.17, 0.08	0.28	23	0.07	−0.09, 0.22	0.40

Bld, blood; *CI*, confidence interval; *ECN*, embryo cell number; *EFS*, embryo fragmentation score

Implantation confirmed by blood hCG tests. Pregnancy confirmed by ultrasound

[1] Adjusted for creatinine levels, age, and cigarette smoking

[2] Negative binomial regression model

[3] Among 33 cases of intracytoplasmic sperm injection

[4] Poisson regression model employing sandwich estimator of the variance

[5] Linear regression model

[6] Excluding $n=1$ influential observation

* Corresponds to false discovery rate (FDR) <0.10

Our results are similar to prior studies that reported no difference in IVF outcomes by cortisol levels, using urine, saliva, or serum levels in addition to self-reported stress questionnaires [13, 25, 26]. Yet, few prior studies employed urine cortisol as a psychological stress biomarker for IVF couples. In one study of 24 h (hr.) urine samples, mean ± = standard deviation cortisol levels for pregnant (222±121 μg/24 h.) and non-pregnant (215±131 μg/24 h.) women using assisted reproduction were similar although higher than levels for an egg donor group (171±40 μg/24 h.) [13]. Another study collected nocturnal urine and first morning voids across three IVF treatment cycle intervals; no significant differences were detected for cortisol in pregnant and non-pregnant women, but an inverse association was described for adrenaline [14]. In contrast, studies of couples conceiving unassisted reported inverse associations between pregnancy and psychological stress measured using salivary alpha-amylase as a biomarker, but not when using salivary cortisol [1, 2]. Additionally, increased stress was associated with early pregnancy loss following unassisted conception in a study utilizing urine cortisol as a biomarker [27]. These results suggest that there may be different roles of the sympathetic nervous system and its products from the adrenal medulla (as measured by salivary α-amylase and adrenaline) and output from the adrenal cortex (as measured by cortisol) for fertility. Overall, we suspect that clinical intervention ameliorates the pejorative effects of psychological stress during IVF, which are reported for couples conceiving unassisted.

We detected significant associations in our determination of toxic metals and cortisol, and also in evaluating the impact of these metals on associations between cortisol and IVF outcomes. Previous evidence suggests toxic metal-cortisol associations in both animals and humans. Among children consuming fish, higher blood Hg levels were associated with lower diurnal cortisol levels [10], and in an experimental rat study cortisol levels increased immediately following Cd treatment [12]. In rainbow trout, Cd treatment decreased cortisol production *via* corticosteroid gene suppression [11]. Although the direction of associations were inconsistent across these few studies, associations between Hg, Cd, and cortisol appear plausible. It may be that these environmental contaminants are interpreted as a stressor (i.e. stimuli that threatens physiological homeostasis); how this interacts with psychological stress is of continued interest.

In a hypothesis-generating exploratory analysis we detected modest evidence for heterogeneity of effects, with an inverse association between cortisol and oocyte fertilization among women with low, but not with high blood Hg. While this result may reflect a chance occurrence, it is also tempting to speculate that higher Hg levels tracked with seafood consumption, and possibly a higher body burden of long-chain n-3 polyunsaturated fatty acids (PUFAs) for which positive reproductive effects have been reported [28]. Earlier work confirmed seafood consumption as the primary source of Hg exposure in our study population [29], which is also a well-recognized source of n-3 PUFAs [30]. In oocytes, PUFAs provide a ready energy source, serve critical cell membrane structural functions, and are biochemical pre-cursors for signaling molecules [31]. Oocyte lipid composition, including PUFA distribution is critical to developmental competence [32].

Differences in lipid composition by grade have been reported for bovine oocytes [33]; oocyte grade is a positive predictor of fertilization [34, 35]. An abundance of n-6 was reported relative to n-3 PUFAs among human oocytes that failed to fertilize [36]. Dietary supplementation with n-3 PUFA has been associated with the increased recovery of high quality oocytes in ewes [37], and greater n-3 PUFA intake has been associated with improved folliculogenesis in agricultural studies [28]. Yet, high-dose n-3 PUFA supplementation reduced oocyte fertilization in mice [38]. Whereas higher pre-cycle n-3 PUFA compromised folliculogenesis, improved embryo morphology was reported for women undergoing IVF with ICSI [39]. Still, no difference was reported in fertilization rate between IVF women following high and low n-3 PUFA diets in one study despite a higher pregnancy rate [40], and a larger ratio of n-3 relative to n-6 PUFAs was associated with lower implantation and pregnancy rates in another study of IVF women [41]. The scenario may be complicated even further by associations between PUFAs and adrenal cortisol synthesis [28]. Unfortunately, we were unable to measure PUFAs in our study and so further investigation is necessary to more definitively interpret our study results.

Our study results should be interpreted cautiously given several limitations. Foremost, the limited sample size precluded simultaneous consideration of women and men, and moreover may have reduced statistical power to detect subtle associations. The QC data suggested increased variability at low cortisol concentrations, which could also have undermined study power secondary to exposure measurement misclassification error, non-differential by outcome. Furthermore, we collected spot urine samples between 9 AM and 1 PM, without a record of the precise collection times for each sample. As such, we were unable to adjust for diurnal variation, which is likely to have further compromised study power. Cortisol follows a circadian rhythm that peaks at approximately 8:30 AM and subsides as the day progresses, reaching a nadir at approximately 12:00 AM [42]. For example, average evening values were approximately 28 % of morning values in a study comparing one hour to 24 h urine collections [43]. Hence, our study would have been better suited to an average of cortisol over repeated urine collections [44], or within a more restricted early morning time frame; however, conducting our study within the usual clinical context of IVF precluded this strategy. Still, a *post hoc* power analysis indicated that 2,762 women were needed to detect the cortisol difference between pregnant versus non-pregnant women. Thus, any potential undetected difference is likely to have been very modest and of questionable clinical relevance.

Despite the aforementioned limitations, our study has several strengths, including its prospective nature, the use of a widely accepted biomarker of psychological stress [45, 46], and highly sensitive biomonitoring methods based on inorganic mass spectrometry for detecting low levels of toxic metals. We considered a spectrum of IVF endpoints associated with the natural history of reproduction, and our exposure assessment was timed approximately to completion of the first oocyte meiosis, following administration of the hCG trigger.

We identified no association between psychological stress and IVF outcomes, using urine cortisol. Though limited, our results could reassure IVF couples and their clinicians that psychological well-being may not have a direct impact on successful IVF treatment. However, our results also raise the possibility for an important role played by toxic metals on potential cortisol-IVF outcomes. More definitive observational research corroborated by use of an experimental animal model will be necessary to elucidate the impact.

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