



# HHS Public Access

Author manuscript

*Int J Environ Health Res.* Author manuscript; available in PMC 2016 January 11.

Published in final edited form as:

*Int J Environ Health Res.* 2015 ; 25(4): 405–417. doi:10.1080/09603123.2014.958137.

## Role of Low Exposure to Metals as Male Reproductive Toxicants

**Dr. H. Anna Jeng,**

Old Dominion University, Norfolk, United States

**Prof. Yeou-Lih Huang,**

Kaohsiung Medical University, Medical Laboratory Science and Biotechnology, Kaohsiung, Taiwan, yelihu@kmu.edu.tw

**Dr. Chih-Hong Pan, and**

Occupational Safety and Health, Occupational Medicine, Taipei County, Taiwan, chpan@mail.ilosh.gov.tw

**Norou Diawara**

Old Dominion University, Norfolk, United States, ndiawara@odu.edu

### Abstract

The objective of the study was to examine the associations between environmentally relevant low metal concentrations and semen quality parameters in men. The concentrations of zinc (Zn), copper (Cu), cadmium (Cd), arsenic (As), selenium (Se) and lead (Pb) in the seminal plasma and urine were measured from 196 male human subjects in Taiwan. Urinary Cd concentrations were negatively associated with sperm viability ( $P=0.006$ ). Seminal plasma Cu concentrations of the normal group ( $15 \times 10^6/\text{ml}$ ) were significantly lower than those of the abnormal group ( $P=0.023$ ). However, the linear regression analysis showed a weak association between Cu concentration and sperm concentration, along with other semen parameters. No significant relationship between other metals (As, Pb, Zn and Se) and semen quality was observed.

### Keywords

Sperm concentration; viability; motility; morphology; metals

### Introduction

Due to the widespread presence of metals in the environment, the general population is exposed to low concentrations of metals through intake of contaminated food and water, contact with contaminated soil, or by breathing in dust or air (Meeker et al., 2008). Metals, e.g. lead (Pb), copper (Cu), zinc (Zn) and cadmium (Cd), have been reportedly linked with alteration of semen quality. Seminal plasma Pb of paint factory workers and the general population adversely affected sperm motility, morphology and concentration (Naha N, 2007; Eibensteiner et al., 2005; Telisman et al., 2007; Meeker et al., 2008). Cd is also among the metals being found to associate with reduced sperm concentration (Colagar et al., 2009; Xu

et al., 2003; Telisman et al., 2000), motility (Xu et al., 2003), and percentages of normal sperm morphology and viability (Colagar et al., 2009; Xu et al., 2003).

Cu is necessary and useful for maintaining good reproductive health since it may act as a cofactor for a variety of important enzymes required for sperm development (Telisman et al., 2000; Huang et al., 2000). Despite its beneficial role, above certain levels, Cu proves to be harmful (Telisman et al., 2000). Similar to Cu, Zn could be beneficial for male reproductive health by acting as one of the factors responsible for antibacterial activity of the seminal plasma or possibly playing a role in sperm production, sperm viability and degradation. Zn deficiency could lead to decreased testicular weight, shrinkage of seminiferous tubules and gonadal dysfunction (Chia et al., 2000). There have been conflicting results about correlations of Zn concentrations in human seminal plasma and semen quality, including sperm concentration, motility and viability (Chia et al., 2000 and Dawson et al., 1998).

To date, human data on some metals has been limited (e.g., As) or inconsistent across studies (e.g., Zn). Also, there is limited data on simultaneously examining semen quality associated with metals in different biological matrixes, such as urine and seminal plasma. Furthermore, there is limited data on the potential role of low, environmentally-relevant exposure to metals in general populations in relation to male reproductive outcomes. This study aimed to examine the association between exposure to low concentrations of trace metals in seminal plasma and urine and semen quality (concentration, morphology, viability and motility) of Taiwanese males in the general population. Semen quality serves as biological endpoints to assess the reproductive status of male individuals in this study and determine potential reproductive toxicity of metals.

## Materials and Methods

### Human subjects

A total of 281 human subjects were screened to determine eligibility for participation in this cross-sectional study. The screening occurred, when they had their annual health examination at the Kaohsiung Municipal Hsiao-Kang Hospital, a main municipal hospital in the southern region of Taiwan. The criteria for selecting human subjects included males between 20 to 65 years old, no medical treatments affecting spermatogenesis (chemotherapy, radiotherapy, and vasectomy), no self-reported diabetes mellitus, cirrhosis or any other liver disease or neoplastic condition, no use of mood stabilizers, medications that affect the endocrine system, and no obesity class II and III according to the National Institutes of Health guidelines (NIH, 2000). The status of fertility was not included as a criterion, since that reproductive status was not the focus of this study. However, we excluded those who had medical issues that prevented them from providing semen voluntarily. A total of 213 human subjects were found eligible and included in this study. The sample size was sufficient to yield a power of 85% with a one-way analysis of variance at the 5% level of significance. All participants were fully informed about the objective of this study and signed the consent form. The participants voluntarily provided semen and a spot of urine. They filled out a questionnaire describing demographic characteristics (age, height, weight, education, and marital status), smoking status, occupational exposure, and

second-hand smoke exposure. Body mass index (BMI) for each human subject was calculated based on height and weight. The study was approved by the Institutional Review Boards at Old Dominion University and Kaohsiung Medical University.

### Semen quality analysis

A semen sample was produced on-site by masturbation into a sterile plastic specimen cup. After collection, the sample was liquefied at 37°C for 20 minutes before analysis. The participants were instructed to abstain from ejaculation for at least three days before semen collection. Each semen sample was collected by masturbation. All semen samples were analyzed for sperm concentration and motility according to World Health Organization guidelines (WHO, 2010). Evaluation of percentage motility was done by manual assessment to quantify percentage of moving sperm compared with non-motile cells for at least 10 microscopic fields. For the morphology assessment, 300 sperm per sample were evaluated from air-dried Papanicolaou-stained preparations and classified as either normal or abnormal according to WHO's strict criteria (WHO, 2010). Results were expressed as percent normal sperm. Total sperm count was calculated as the product between sperm concentration and ejaculate volume. Total motile sperm was calculated as the product between total sperm count and progressive motility. All of the procedures were performed by the same laboratory technician. The mean inter-individual coefficients of variation were 18.2, 12.3, and 15.2% for sperm concentration, motility, and viability, respectively

### Determination of metals in seminal plasma

The semen samples were centrifuged at 1,000 rpm for 5 minutes to separate sperm and seminal plasma. Then, 300 µL of each seminal plasma sample was transferred into a pre-cleaned 1.5 mL microtube and treated with 300 µL concentrated nitric acid at room temperature for 20 minutes. After homogenization, the mixture was diluted 5, 5, 1, 200 and 5 fold with deionized water for Cd, Cu, Pb, Zn and As, respectively. After vigorous shaking, the sample solution was centrifuged at 12,000 rpm for 5 minutes. The obtained supernatant was directly analyzed by flame atomic absorption spectrometry (FAAS) for Zn, electrothermal atomic absorption spectrometry (ETAAS) for Cu, Cd, Se and Pb, and hydride generation atomic absorption spectrometry (HGAAS) for As. The accuracy was validated by the standard spiked method, and the As, Cd, Cu, Pb, Se and Zn concentrations in spiked semen samples were obtained by similar analytical methods. Recovery of the target analytes in spiked semen ranged from 95.10 to 105.97%. The calibration curves were obtained after analyzing the standard series by FAAS, ETAAS, HGAAS as described, respectively. A good linearity was obtained at the concentration range of 5-30 ( $r^2=0.9973$ ), 0.5-2.5 ( $r^2=0.9999$ ), 5-50 ( $r^2=0.9990$ ), 5-60 ( $r^2=0.9997$ ), 1-6 ( $r^2=0.9991$ ) and 250-1000 ( $r^2=0.9989$ ) µg/L for As, Cd, Cu, Pb, Se and Zn respectively. Limits of detection (LOD) for As, Cd, Cu, Pb, Se and Zn were 2.59, 0.03, 1.03, 0.98, 0.02 and 0.065 µg/L. Limits of quantitation (LOQ) for As, Cd, Cu, Pb, Se and Zn were 8.46, 0.09, 2.95, 3.14, 0.07 and 0.217 µg/L, respectively. The concentrations of metals in seminal plasma were expressed as µg/L.

### Determination of metals in urine

The urine samples were prepared as follows: 500 µL of urine was transferred into a pre-cleaned 4 mL microtube and treated with aliquot of concentrated nitric acid at room

temperature for 20 minutes. Then, the mixture solution was diluted 1, 1, 1 and 200 fold with deionized water for Cd, Cu, Pb and Zn, respectively. After vigorous shaking, the urine samples were directly analyzed by FAAS for Zn, ETAAS for Cu, Cd, Pb and Se. For As, urine samples were filtrated using a 0.45  $\mu\text{m}$  hydrophilic polyethersulfone membrane prior to measurement by HGAAS. The accuracy was validated by determining the contents of As, Cd, Cu, Pb, Se and Zn in standard reference material NIST-2670a human urine. The urine SRM 2670a was diluted similarly prior to analysis and the recoveries ranged from 93.05 to 108.17%. The accuracy was also validated by the standard spiked method, with the As, Cd, Cu, Pb, Se and Zn concentrations in spiked semen samples obtained by similar analytical methods. Recovery of the target analytes in spiked semen ranged from 93.05 to 104.91%. The calibration curves were obtained at the concentration range of 5-60 ( $r^2=0.9976$ ), 1-5 ( $r^2=0.9998$ ), 10-80 ( $r^2=0.9991$ ), 10-90 ( $r^2=0.9994$ ), 20-100 ( $r^2=0.9991$ ) and 100-800 ( $r^2=0.9999$ )  $\mu\text{g/L}$  for As, Cd, Cu, Pb, Se and Zn, respectively. The concentrations of As, Cd, Cu, Zn, Pb and Se in urine samples were obtained by the general standard curve method. Concentrations of metals were adjusted by using urinary creatinine concentrations and expressed as ng/g creatinine. LOD for As, Cd, Cu, Pb, Se and Zn were 2.59, 0.03, 1.03, 0.98, 0.02, and 0.065  $\mu\text{g/L}$ , respectively. LOQ for As, Cd, Cu, Pb, Se and Zn were 8.46, 0.09, 2.95, 3.14, 0.07, 0.217  $\mu\text{g/L}$ , respectively. Urine volumes were adjusted by creatinine levels. The concentrations of metals were expressed as  $\mu\text{g/g}$  of creatinine.

### Statistical Analysis

Descriptive analysis was conducted to determine the mean, distribution of demographics, semen quality parameters, and metal concentration in seminal plasma and urine. Mean concentration of each metal was calculated based on two separate groups of each semen parameter based on the WHO reference values. Mean metal concentrations in two categories of each semen parameter were compared using the Student's *t-test* after ascertaining homogeneity of variance between the two groups. Association between exposure categories for each metal and sperm concentration, motility and vitality was assessed by binary logistic regression, where subjects were dichotomized in categories less than or greater than WHO reference levels (concentration  $>15 \times 10^6/\text{mL}$ , motility 40% and vitality 58%) (WHO, 2010). Age, drinking and smoking were considered as the confounding factors. We then constructed a full multivariable linear regression model for each semen quality parameter as the dependent variable and individual ln-transformed As, Zn, Cd, Cu, and Pb as a predictor along with age (continuous variable), smoking (dichotomous), and drinking (dichotomous), BMI (continuous), and ln-transformed urinary creatinine (continuous) as covariates. Regression results are presented as the change in semen quality measure associated with a unit increase in ln-transformed metal concentration. Data analysis was performed using SPSS for Windows (version 22).

### Results

Table 1 summarizes demographic characteristics of the participants stratified by semen quality per WHO reference value categorizations. Among 196 participants, a total of 62 human subjects with semen parameters (concentration, motility, viability, and motility) met WHO reference values. All participants were Asian, with a mean age of  $38 \pm 10$  years and

BMI of  $25 \pm 4.1$  kg/m<sup>2</sup>. Almost 50% of participants did not smoke as well as did not drink. There was a difference in percentage of drinking between those with normal motility and those below normal motility. But none of the variances was statistically significant ( $P=0.11$ ).

Table 2 describes the concentration of metals in seminal plasma and urine. Seminal plasma Pb and As concentrations were respectively in 79% and 51% of samples below the LOD. Seminal plasma Cu and Se concentrations in all the samples were detectable, while Cd concentrations in 17% of the samples were below the LOD. Urinary metals, e.g. Zn, Cd, Cu and As, in most urine samples (>98.5%) were detectable, with 4.2% of urinary Pb levels contained in 4.2% of samples below the LOD.

Tables 3 and 4 summarize comparison of metal concentrations in two categories of each semen parameter, based on the cut points suggested by the WHO to determine normal vs abnormal condition for each semen parameter. All metals in seminal plasma and urine with and without creatinine adjustment were not significantly different in the two categories of the semen parameters except Cu in seminal plasma and As in urine with creatinine adjustment in relation to sperm concentration. Average Cu concentration in seminal plasma with sperm concentration  $<15 \times 10^6$ /mL was higher than that in sperm concentration  $15 \times 10^6$ /mL ( $P=0.023$ ). Average As in urine with creatinine adjustment in sperm concentration  $<15 \times 10^6$ /mL was significantly higher than that in sperm concentration  $15 \times 10^6$ /mL ( $P=0.003$ ).

Table 5 shows a linear analysis of regression between semen parameters and metals after controlling for covariates, including age, smoking, drinking, and BMI. Cd concentrations in urine had negative correlations with viability ( $r = -0.216$ ,  $P=0.006$ ;  $-r=0.301$ ,  $P=0.001$ ), but there was no correlation between the seminal plasma Cd concentrations and all of the semen analysis parameters. Also, we did examine correlations of covariates and semen quality. All of the covariates did not correlate with semen quality except age ( $P=0.045$ ). In addition, we obtained personal habits, including hot showers and bathes and exercise, from the questionnaire. The frequency of hot showers and bathes and exercise, however, did not associate with semen quality.

After further examining the correlation between semen parameters and metal concentrations, the stepwise multiple regression analysis was conducted by including covariates (age, BMI, smoking and drinking). The stepwise selection approach included BMI and seminal plasma Cd using the best fit regression model in relation to sperm concentration. Those parameters could explain the variations in sperm concentration. For motility, age and BMI were included in the best fit regression model, while no metals were included. Age and urinary Cd were included in the best fit regression related to percentage of sperm viability (Table 6).

## Discussion

Experimental animal and human occupational studies generally support an adverse role for high exposure levels of metals in human reproductive health. However, evidence on the effects of low, environmentally-relevant exposure levels of metals on male reproductive outcomes is still limited. In the present study, we measured metal concentrations in seminal

plasma and urine from a general population group in Taiwan. Detected metal concentrations were compared with the findings from other studies on low exposure levels of metals (Telisman et al., 2007), which were lower than those metal concentrations in workers through occupational exposure.

The present study simultaneously measured metal concentrations to examine correlations of metals between urine and seminal plasma. Limited studies have reported correlations of metals in blood and seminal plasma. However, there was inconsistency about whether those metals in seminal plasma correlated with those in blood (Benoff et al., 2009; Chia et al., 2000; Wong et al., 2001). Currently, there are very limited references about examining the relationship of metals in seminal plasma and urine in the context of assessment of male reproductive health. In the present study, As was the only metal with a correlation between the measurements in seminal plasma and urine ( $P = 0.001$ ). Similar findings were observed in the comparison with blood and seminal plasma. The finding may suggest that urinary As could represent seminal plasma As to serve as an indicator for assessing the effect of As on male reproductive health. With the observation of no significant correlation for other metals (Pb, Zn, Cu and Cd), it raises the need for a better understanding of metal metabolisms and kinetics in association with the biological structure of the male reproductive system. Such information could be relevant for selecting an appropriate biomarker for biomonitoring of metal exposure associated with male reproductive health and assessing the target organs and tissues and mechanisms of metal-induced reproductive toxicity in men.

Cd levels in human biological samples associated with environmental exposure have been measured to assess reproductive toxicity of this metal in men (Li et al., 2012; Xu et al., 2003; Keck et al., 1995). The results proved to be inconsistent. The different findings could stem from selection of human subjects (smokers, general population, and infertility in patients), sample sizes, metal concentrations, and adjustment for potential confounders. The present study showed that Cd levels in a group from the general population were negatively associated with sperm viability. Cd, along with age, was included in the best fit regression model that explained variations in percentage of sperm viability. The association continued to exist in separate examinations of smokers and nonsmokers. Cd levels in seminal plasma were compared with the levels in nonsmokers from a general population group as reported by other studies (Keck et al. 1995) and were lower than those in infertile smokers (Xu et al., 2012; Telisman et al., 2007). Our research finding added evidence that low exposure to Cd in the general population could link to decreased sperm quality, particularly in viability. Literature on the subject has reported likely biological mechanisms, including altering homeostasis of reproductive hormones via the steroidogenic pathway and oxidative stress due to a decrease in the ability of sulfhydryl molecules to scavenge reactive oxygen species (Jahan et al., 2014; Ren et al., 2012).

Cu content is important for maintaining proper reproductive functions. However, high Cu levels could be harmful to reproductive functions. Cu levels detected in our study showed an inverse association with sperm concentration. Also, Cu levels in the seminal plasma of the normal group ( $15 \times 10^6/\text{mL}$ ) were significantly lower than those of the abnormal group ( $P=0.023$ ) (Table 3). A cross-sectional study of 23 and 39-year-old men attending the Reproductive Medicine Center in China also observed that Cu levels were negatively linked



with sperm concentration (Li et al., 2012). Average Cu level in seminal plasma (208 µg/ml) from Li's study was higher but comparable with the Cu level (170.8 µg/ml) in seminal plasma) in the present study. Besides sperm concentration, we did not observe that Cu was associated with other semen parameters, including motility, viability, and morphology. However, accumulative studies have documented that Cu levels in biological media (seminal plasma, urine, and blood) could decrease sperm motility (Wong et al., 2001), which may be critical for spermatogenesis and fertility. In its ionic form ( $\text{Cu}^{+2}$ ), Cu could affect the fertilizing capacity of human gametes *in vitro* and interfere with the sperm-oocyte interaction leading to fertilization (Roblero et al., 1996). Also, the involvement of Cu on superoxide dismutase could have an influence on sperm motility and viability, which are the important factors in semen fertility (Garratt et al., 2013; Eghbali et al., 2008).

To date, evidence regarding the effects of low level arsenic exposure on human male reproductive outcomes is still scarce. We observed there was a weak relationship between As and semen parameters after adjusting for smoking, age, and BMI. This finding contrasted to other studies showing an inverse association between low sperm motility and exposure to environmental levels of As (Meeker et al., 2008; Xu et al., 2012). Also, As has been recognized as an endocrine disruptor since it alters steroid and thyroid hormone receptor-mediated gene regulation (Davey et al., 2008; Jana et al., 2006). More research is needed to confirm the role of exposure to As at a low, environmentally-relevant concentration on semen quality and any underlying mechanisms attributed to As reproductive toxicity. In comparing detected As levels in the present study, the present study recorded lower As levels as compared to those in other studies, which reported a significant impact of As on semen quality (Meeker et al., 2008; Xu et al., 2012). A similar situation occurred in the association between Pb and semen quality. The study population had low Pb levels in seminal plasma and urine, particularly those with healthy sperm concentration having Pb levels below detection limits. Seminal plasma Pb concentrations in the study were lower than other studies, which also include general populations with low environmental exposure (Hernández-Ochoa et al., 2005; Morán-Martínez et al., 2013). They report interesting and controversial results; for example, Hernández-Ochoa *et al.*, did not find a significant correlation between exposure to Pb (2.2 µg/L) and semen quality. However, Morán-Martínez reported that chronic environmental exposure to Pb (3.28 µg/L) affected semen quality in a Mexican male population based on comparisons of the exposure group to the non-exposed group (Morán-Martínez et al., 2013).

Smokers in the present study had slight higher Pb and As concentrations (175.4 and 4.54 µg/L in seminal plasma, respectively) than those of nonsmokers (164.6 and 4.49 µg/L in seminal plasma, respectively). That indicates that smoking was not the main source of the metals. About 51% of study subjects smoked in this study. However, they only smoked 2-3 cigarettes/day, which may not sufficient to significantly elevate metal concentrations in seminal plasma and urine in humans.

Epidemiological studies have shown that occupational exposure to high Pb levels could lead to alterations in semen quality (Eibensteiner et al., 2005). Possible underlying mechanisms may be via altering gonadotropins, which bind to specific receptors that lead to impair spermatogenesis and gamete growth (Benoff et al., 2000). Also, the same process may lead

to altered hypothalamic-pituitary-gonadal function in relation to an imbalance in hormone hormonal enzyme activities and hormone secretion (Benoff et al., 2000). Recent studies have been encouraged to investigate the reproductive adverse effects of Pb at environmentally relevant low levels in biological fluids and tissue levels. Low lead exposure levels have been associated with adverse human male reproductive effects in several studies (Telisman et al., 2000; Telisman et al., 2007), but conclusions have been less consistent than the high exposure to Pb. A study recruited men from infertility clinics in Michigan, USA to assess the effects of environmental concentrations of metal exposure on measures of male reproduction. Blood Pb concentration of 15 µg/L did not associate with sperm concentration, motility, or morphology in models adjusted for age and current smoking. The insignificant relationship observed in our study may stem from low Pb levels. The mean concentrations for Pb in urine and seminal plasma were 5.4 µg/L and 0.6 µg/L, respectively. A cross-sectional study of 341 Taiwanese infertile males with no occupational exposure reported a mean level of 2.19 µg/L of Pb in seminal plasma, which was approximately four times as high as the Pb concentration detected in the present study. At the detected mean concentration, seminal plasma Pb inversely correlated with sperm count, but not with motility or morphology (Wu et al., 2012). Although there is a growing interest in determining the reproductive effects from low exposure to metals, there is, however, a lack of data to establish a threshold level to determine the lowest level for adverse effects of lead concentration on semen quality. Also, underlying mechanisms for low exposure of Pb is unclear, although a hypothesis has been proposed that lead-detrimental spermatogenesis rather than lead-altered hypothalamic-pituitary-gonadal function could contribute to the association between low lead concentration in semen and sperm count (Allouche et al., 2009).

When examining the correlation between metals and semen quality, covariates (age, BMI, smoking, and drinking) were controlled. In an attempt to develop a model to explain the variation in semen quality, those covariates were included in the consideration. The stepwise regression analysis revealed that age and BMI were negatively associated with motility and viability. The obesity epidemic remains a growing public health concern, with an increasing interest in understanding how obesity impairs reproductive health. Some studies have suggested that an elevated male BMI can lead to impaired sperm production (Eisenberg et al., 2014; Macdonald et al., 2013). Our study showed that BMI negatively associated with sperm concentration, motility and viability. Mean BMI of the studied population was 25.4. A majority of the human subjects had BMI in the healthy weight range. Possible etiology explaining the relationship between adiposity and poor sperm quality include alterations in the hypothalamic-pituitary-gonadal axis and elevated scrotal temperatures due to insulation of the scrotum caused by abdominal adipose tissue (Michalakis et al., 2013)

In conclusion, we found some suggestive or significant relationships between metals and semen quality in the present study. The most consistent evidence existed for an inverse association between Cd and viability, even when potential confounding variables were simultaneously considered. BMI and age also exhibited an inverse relationship with motility and viability and/or concentration of sperm.



## Acknowledgments

The project was partially supported by a grant (1R15ES018952-01) from the U.S. National Institute of Environmental Health Sciences.

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**Table 1**

Demographic characteristics of the participants by semen quality parameters classified according to the WHO reference levels.

	All	Comparison group* N= 62	Concentration <20 × 10 <sup>6</sup> /ml N= 6	Motility <40% N= 31	Vitality <58% N= 21	Normal morphology <4% N= 66
Age (years, mean ± SD)	38.4 ± 9.9	36.8 ± 9.7	35.7 ± 11.6	43.8 ± 9.4	46.1 ± 8.6	38.7 ± 10.0
BMI (kg/m <sup>2</sup> , mean ± SD)	25.0 ± 4.1	24.8 ± 3.9	24.2 ± 1.8	25.6 ± 4.4	25.9 ± 4.1	25.1 ± 4.4
Smoking status (%)						
Yes	50.9	47.6	50.0	48.6	50.0	60.6
No	49.1	52.4	50.0	51.4	50.0	39.4
Drinking (%)						
Yes	30.5	28.6	24.2	20.6	23.8	36.9
Yes/Quit	2.4	1.6	1.3	2.9	4.8	3.1
No	67.1	69.8	74.5	76.5	71.4	60.0
Education (%)						
<High school	30.5	19.3	33.3	48.6	61.9	45.5
High school	49.4	56.5	16.7	40.0	23.8	39.4
College/Post College	20.1	24.2	50.0	11.4	14.3	15.2

\* The comparison group was comprised of men with all four semen parameters higher than the reference levels according to the WHO reference levels.

**Table 2**

Distribution of semen quality and metals in seminal plasma, urine, and urine adjusted for creatinine levels.

	All	10 <sup>th</sup>	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	90 <sup>th</sup>	95 <sup>th</sup>	Maximum
Metal concentration in seminal plasma (µg/L)								
Zn	169.3 ± 97.9	70.6	101.0	140.0	208.8	304.6	371.2	612.0
Cd	0.5 ± 0.3	0.38	0.4	0.6	1.4	1.5	1.5	1.5
Cu	170.8 ± 210.1	66.9	81.7	116.2	170.4	252.2	440.1	1756.8
As	4.8 ± 5.5	1.3	1.3	1.3	7.3	12.7	16.3	32.8
Se	249.1 ± 89.1	132.1	174.9	247.1	315.2	353.3	362.5	609.7
Pb	0.6 ± 1.1	0.5	0.5	0.5	0.5	0.5	0.5	12.6
Metal concentration in urine (µg/L)								
Zn	602.0 ± 576.0	98.2	176.0	447.2	849.4	1414.3	1669.3	3279.6
Cd	0.7 ± 0.6	0.2	0.4	0.9	1.4	2.4	5.8	2.9
Cu	14.8 ± 13.9	3.1	5.2	11.5	20.3	31.8	36.1	94.1
As	133.2 ± 146.4	12.7	33.3	77.1	177.6	336.1	433.8	754.5
Se	20.4 ± 13.8	8.3	12.4	17.4	24.7	35.6	45.7	84.3
Pb	5.4 ± 6.4	0.3	0.5	3.1	8.5	15.1	17.8	34.7
Metal concentration in urine adjusted for creatinine (µg/g creatinine)								
Zn	419.0 ± 345.8	147.2	244.7	349.4	502.0	707.8	819.7	2938
Cd	0.5 ± 0.4	0.1	0.2	0.4	0.8	1.1	1.4	2
Cu	12.3 ± 11.6	3.8	6.0	10.1	13.8	22.1	32.5	88
As	91.1 ± 91.9	19.4	37.4	64.1	110.8	184.5	297.7	686
Se	15.4 ± 10.8	5.7	7.5	12.6	20.3	31.0	39.3	58
Pb	4.4 ± 7.8	0.2	0.8	2.5	6.1	8.5	12.1	72
Semen quality								
Concentration (10 <sup>6</sup> /ml)	148.1 ± 134.0	34.8	60.0	111.5	190.0	273.0	313.5	905.0
Motility (% motile)	58.4 ± 22.6	21.9	43.1	61.3	75.3	86.3	89.0	99.0
Morphology (% normal)	4.8 ± 3.8	0.5	2.0	4.0	7.0	10.9	12.2	19.0
Viability (%)	80.9 ± 18.9	50.2	71.5	86.0	96.9	99.0	99.6	99.9

**Table 3**

Metals in seminal plasma classified by semen parameters according to the WHO reference levels.

	Cu	Zn	Pb	As	Cd	Se
Sperm concentration						
15×10 <sup>6</sup> /ml	137.8 ± 210.1	144.3 ± 103.2	0.6 ± 1.1	7.7 ± 5.7	0.5 ± 0.3	122.6 ± 128.0
<15×10 <sup>6</sup> /ml	303.8 ± 258.8	181.4 ± 183.8	<DOL	10.3 ± 10.3	0.8 ± 0.2	179.1 ± 140.3
P value	0.023	0.345	n/a	0.54	0.190	0.267
Motility						
40%	145.6 ± 307.5	141.1 ± 102.3	0.6 ± 0.4	6.8 ± 5.1	0.4 ± 0.4	96.1 ± 151.0
<40%	146.4 ± 164.8	149.2 ± 106.4	0.7 ± 1.2	8.2 ± 6.1	0.5 ± 0.3	133.1 ± 136.4
P values	0.976	0.803	0.892	0.305	0.375	0.164
Viability						
58%	160.6 ± 297.9	142.1 ± 101.4	0.6 ± 1.2	6.4 ± 4.7	0.4 ± 0.3	101.1 ± 113.2
<58%	143.0 ± 183.0	146.7 ± 109.5	0.6 ± 0.4	8.0 ± 5.9	0.5 ± 0.3	140.1 ± 133.7
P values	0.697	0.866	0.956	0.377	0.358	0.126
Normal morphology						
4%	143.4 ± 182.1	148.0 ± 124.5	0.7 ± 1.4	6.9 ± 5.6	0.5 ± 0.4	110.3 ± 142.9
<4%	146.9 ± 220.5	144.5 ± 91.9	0.5 ± 0.2	8.6 ± 6.0	0.5 ± 0.3	138.9 ± 136.5
P values	0.940	0.840	0.547	0.301	0.237	0.188

&lt;DOL: Detection of limit

\*N/A: A majority of samples had Pb values below detection limit.

Metal concentration in µg/L

**Table 4**

Metals in urine classified by semen parameters according to the WHO reference levels

	Cu	Zn	Pb	As	Cd	Se
<sup>a</sup> Metal in Urine						
Concentration						
15×10 <sup>6</sup> /ml	11.4 ± 13.8	445.2 ± 570.8	4.2 ± 5.82	95.7 ± 137.8	0.5 ± 0.6	9.3 ± 0.6
<15×10 <sup>6</sup> /ml	12.3 ± 8.8	438.6 ± 279.0	7.7 ± 8.71	172.8 ± 141.1	0.9 ± 0.6	14.2 ± 14.7
P-Value	0.854	0.974	0.109	0.124	0.09	0.301
Motility						
40%	11.0 ± 13.9	299.2 ± 376.4	3.3 ± 4.9	100.7 ± 184.8	0.6 ± 0.8	7.5 ± 15.6
<40%	11.5 ± 13.5	481.9 ± 592.6	4.7 ± 6.3	98.9 ± 124.3	0.5 ± 0.5	10.1 ± 12.3
P-Value	0.822	0.09	0.245	0.949	0.871	0.301
Viability						
58%	12.8 ± 15.2	360.8 ± 401.5	4.1 ± 5.2	122.9 ± 202.7	0.8 ± 0.9	11.5 ± 18.5
<58%	11.2 ± 13.3	458.7 ± 581.3	5.8 ± 7.5	95.4 ± 125.2	0.5 ± 0.5	9.2 ± 11.9
P-Value	0.587	0.440	0.199	0.369	0.09	0.430
Normal morphology						
4%	10.0 ± 11.8	439.6 ± 568.5	3.4 ± 4.9	97.8 ± 134.9	0.5 ± 0.6	9.7 ± 15.5
<4%	11.8 ± 15.4	449.6 ± 554.7	4.7 ± 6.3	100.7 ± 134.9	0.6 ± 0.6	9.4 ± 10.5
P value	0.70	0.91	0.268	0.870	0.840	0.901
<sup>b</sup> Metals in urine with adjusted creatinine levels						
Concentration						
20×10 <sup>6</sup> /ml	11.2 ± 11.6	287.5 ± 349.5	4.2 ± 7.8	58.8 ± 81.9	0.4 ± 0.4	6.4 ± 10.8
<20×10 <sup>6</sup> /ml	12.4 ± 7.6	301.9 ± 223.2	8.5 ± 4.6	152.7 ± 104.9	0.6 ± 0.5	10.9 ± 11.3
P value	0.81	0.908	0.145	0.0026	0.127	0.192
Motility						
40%	8.9 ± 15.3	246.9 ± 336.7	2.2 ± 3.7	70.5 ± 141	0.4 ± 0.4	4.1 ± 6.4
<40%	8.5 ± 9.4	298.6 ± 346.6	3.4 ± 7.3	61.3 ± 66.0	0.5 ± 0.4	7.3 ± 8.0
P value	0.860	0.443	0.343	0.580	0.515	0.07
Viability						
58%	6.5 ± 6.8	230.1 ± 265.5	3.9 ± 6.7	58.8 ± 79.2	0.4 ± 0.4	5.7 ± 7.3
<58%	8.9 ± 11.5	297.7 ± 355.3	4.1 ± 6.6	67.2 ± 93.3	0.8 ± 0.4	6.8 ± 9.8
P value	0.300	0.383	0.587	0.530	0.050	0.590
Normal morphology						
4%	7.9 ± 8.9	272.2 ± 274.5	2.6 ± 3.9	73.9 ± 149.8	0.4 ± 0.4	5.5 ± 8.1
<4%	9.1 ± 12.5	302.3 ± 397.0	3.7 ± 8.5	61.5 ± 71.7	0.7 ± 0.3	7.7 ± 10.5
P value	0.510	0.578	0.290	0.515	0.090	0.140

<sup>a</sup>Metal concentration in µg/L<sup>b</sup>Metal concentration in µg/L creatinine



**Table 5**

The Pearson Product Moment correlation coefficient and the level of significance (r,p) for relationships between semen quality and metal concentrations in urine and urine adjusted for creatinine with controlling for smoking, age, drinking, an BMI<sup>a</sup>

	Concentration	Motility	Viability	Normal Morphology
Metals in seminal plasma				
Cd	-0.092 (0.302)	-0.0751 (0.396)	-0.002 (0.981)	-0.142 (0.118)
Cu	-0.128 (0.106)	-0.0176 (0.825)	-0.0873 (0.272)	-0.0769 (0.348)
Zn	-0.081 (0.309)	-0.089 (0.261)	-0.0281 (0.725)	-0.0563 (0.494)
As	-0.114 (0.313)	-0.0373 (0.741)	-0.0912 (0.418)	-0.109 (0.361)
Se	-0.166 (0.053)	0.376 (0.056)	-0.263 (0.060)	0.161 (0.050)
Metals in urine				
Cd	-0.153 (0.051)	-0.026 (0.746)	-0.216 (0.006)	-0.091 (0.266)
Cu	-0.057 (0.467)	-0.04 (0.612)	-0.066 (0.414)	-0.0436 (0.582)
Pb	-0.187 (0.018)	-0.061 (0.44)	-0.090 (0.26)	0.078 (0.343)
Zn	-0.048 (0.549)	0.097 (0.219)	0.0183 (0.818)	0.0997 (0.222)
As	-0.163 (0.0362)	0.0419 (0.591)	-0.096 (0.217)	-0.087 (0.28)
Se	-0.092 (0.221)	0.101 (0.410)	0.090 (0.302)	0.018 (0.903)
Metals in Urine/Creatinine				
Cd	-0.170 (0.087)	-0.170 (0.087)	-0.319 (0.001)	-0.003 (0.981)
Cu	-0.057 (0.568)	-0.065 (0.515)	-0.117 (0.239)	-0.023 (0.822)
Pb	-0.456 (0.09)	-0.031 (0.757)	-0.034 (0.213)	-0.004 (0.970)
Zn	-0.046 (0.646)	-0.082 (0.407)	-0.088 (0.376)	-0.036 (0.730)
As	-0.345 (0.09)	0.099 (0.310)	-0.144 (0.138)	0.114 (0.263)
Se	-0.133 (0.321)	0.109 (0.417)	0.093 (0.487)	0.011 (0.933)

Pb was not included due to more than 45% of the samples with a deflection limit.

<sup>a</sup>Semen quality parameters and metal concentrations were ln-transformed.

**Table 6**

The stepwise multiple regression results for relationships between each semen parameter considered and all metals (Pb, Cd, Zn, Cu and As), alcohol consumption, age, and BMI.<sup>a</sup>

<b>Dependent Parameters</b>	<b>Independent parameters</b>	<b>Parameter estimates</b>	<b>Standard errors</b>	<b>P</b>
Concentration	BMI	-0.0648	0.003	<0.001
	Cd, seminal plasma	-0.002	0.016	0.056
Motility	Age	-0.021	0.0044	<0.001
	BMI	-0.011	0.0101	0.295
Viability	Age	-0.004	0.0024	<0.001
	BMI	0.0005	0.005	0.920
	Cd, urine	-0.001	0.001	0.397

<sup>a</sup>Semen quality parameters and metal concentrations were ln-transformed. No data transformation was needed for age and BMI.