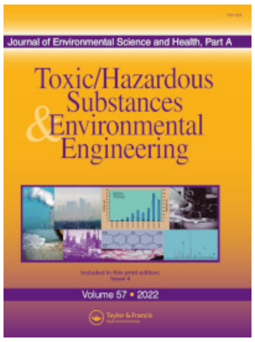

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Journal of Environmental Science and Health, Part A

Toxic/Hazardous Substances and Environmental Engineering

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lesa20

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To cite this article: Hueiwang Anna Jeng, Sinjini Sikdar, Yeou-Lih Huang & Chih-Hon Pan (2022) Mixture analysis of associations between exposure to low levels of multiple metals and semen quality and sperm DNA integrity, Journal of Environmental Science and Health, Part A, 57:4, 318-326, DOI: 10.1080/10934529.2022.2061256

To link to this article: https://doi.org/10.1080/10934529.2022.2061256

Published online: 07 Apr 2022.

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART A
2022, VOL. 57, NO. 4, 318–326
https://doi.org/10.1080/10934529.2022.2061256

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Mixture analysis of associations between exposure to low levels of multiple metals and semen quality and sperm DNA integrity

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ABSTRACT

The objective of this study was to assess the association of exposure to metal mixtures with semen quality and sperm DNA integrity of coke oven workers ($n = 96$). Urinary six metals (cadmium, lead, arsenic, zinc, selenium, and copper) were quantified using inductively coupled-mass spectrometry. Semen quality parameters included sperm concentration, sperm concentration, sperm motility, sperm morphology, and sperm viability. Sperm DNA fragmentation and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) adducts served as biomarkers for assessing sperm DNA integrity. Bayesian kernel machine regression with the hierarchical variable selection process was used for analyzing both individual and joint effects of the metal mixture on the outcomes of semen samples, while adjusting for age, smoking, alcohol consumption, job length, and body mass index. The metal mixture was associated with reduced sperm concentration, motility, viability, and normal morphology. It was novel that a dose-response relationship was observed between exposure of the metal mixture and semen quality. Among the metals tested, cadmium had a reverse relationship with sperm motility, viability, and normal morphology, and a non-linear relationship with sperm viability and sperm motility. The metal mixture and individual metals were not associated with sperm DNA fragmentation and 8-oxodGuo. In conclusion, exposure to metal mixtures and cadmium may exert an association with semen quality and had no association with sperm DNA breakages.

ARTICLE HISTORY
Received 21 December 2021
Accepted 22 March 2022

KEYWORDS
Metals; mixture analysis; semen quality; cadmium

Introduction

Recent meta-analysis analyses revealed that sperm counts among men in North America and other countries decline more than 59% from 1973 to 2011.[1,2] Also, a parallel increase in the incidence of genitourinary abnormalities has been recorded.[3] A growing number of studies reported that declined sperm count and male reproductive function have been plausibly associated with environmental exposure and lifestyle factors.[4–8]

Exposure to metals have been reported to link to semen quality. Cadmium (Cd) has been recognized for its reproductive toxicity with an association with impaired testicular function,[9] decreased sperm motility,[10–12] decreased sperm normal morphology,[13] and increased sperm DNA damage.[14,15] Also, lead (Pb) has linked to decreased sperm normal morphology, motility, and DNA fragmentation.[15,16] Essential metals, such as chromium (Cr), copper (Cu), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn), are crucial for the function of biological systems, but they may be harmful to male reproductive functions above certain levels.[17]

Humans are exposed to multiple metals at varied levels and from varied sources. Currently, very few studies have conducted mixture analyses of metal mixtures with low levels of individual metals on adverse semen quality outcomes. Although metals tend to interact in biological reactions and activities, limited studies, however, examined interactions among metals. Consideration of joint effects of multiple pollutants may advance our understanding of male reproductive health effects of metal pollution. Within this context, we used Bayesian kernel machine regression (BKMR) modeling to evaluate the associations between exposure to multiple metals and semen quality outcomes.[18,19] The BKMR modeling approach with the hierarchical variable selection process was proposed to estimate overall effect while accounting for possible interactions of non-linearity and non-additives between metals and semen quality endpoints, and to quantify the magnitude or direction of associations between metal components and semen quality outcomes.

Materials and methods

Subjects

Participants were coke oven workers ($n = 93$) who were employed at the largest steel plant in Southern Taiwan. They were full-time employees working on the coking

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processes in the topside-oven and site-oven areas. Our ongoing environmental monitoring showed increased levels of metals in the coke oven surrounding areas, as compared to the background level.[6,7] Eligibility criteria of human subjects included more than five years of employment in the coke oven processing, ages between 25 to 60 years old to reduce an age effect on semen quality, no reproductive dysfunction (normal ejaculatory function and fertility), and no history of cancer of the male reproductive system. We screened the entire roster of the plant to determine subjects' eligibility at their mandatory annual health checkup and then randomly selected subjects from the pool of eligible subjects for this study. All the subjects filled out a questionnaire and provided semen and urine samples. A self-administered questionnaire was designed to collect information pertaining to demographics and covariates. Demographic variables include age (continuous), education level ($\leq$high school, $\geq$ high school). Smoking (Yes/No), alcohol consumption (Yes/No), weight (continuous), and high (continuous) were reported. Questions on employment history emphasized current occupational duties, production processes, respirator usage, and job classification. Health history covered the participant's and family's history of cancer, other diseases, and treatment history. Dietary intake included seafood and rice items. The study was approved by the Institute of Research Board at Old Dominion University. All participants were fully informed about the objective of the study and signed the consent forms.

Urinary metals

Urine samples were collected for quantifying the internal human exposure dose of the metal mixture, including As, Cd, Pb, Cu, Se, and Zn. We selected the metals based on the preliminary results from our environmental monitoring.[6,7] Blank samples were collected and quantified for the metal mixture to ensure no contamination of the collection vessel. The urine samples were pretreated using an acid digestion procedure 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 analysis of Cd, Pb, Cu, Se, and Zn, respectively. The metals in the mixture solution were quantified by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-MS) (Thermo Scientific, Waltham, MA, USA). 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 levels 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 level 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) µg/L for As, Cd, Cu, Pb, Se and Zn, respectively. Levels of As, Cd, Cu, Zn, Pb and Se in urine samples were obtained by the general standard curve method. 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, respectively. Urine volumes were adjusted by creatinine levels. The levels of metals were expressed as µg/L.

Semen collection and semen quality analysis

The participants were instructed to abstain from ejaculation for at least three days before semen collection. At the end of a work shift, workers arrived at the Kaohsiung Municipal Hsiao Kang Hospital where they had annual health examination and collected their semen samples via ejaculation. Within one hour after the sampling of ejaculate, a technician conducted all routine semen quality analysis in the laboratory of the hospital following the World Health Organization guidelines, along with quality control and assurance procedures.[20] All laboratory tests were done in a blind fashion. Another technician also assessed one semen sample/10 samples to determine accuracy and consistency of the analysis and outcomes. When sperm counts are less than 15×10^6/ml, the condition is recorded as oligospermia. Percentage of oligospermia is quantified as A/T, where A equals the number of sperm count $< 15 \times 10^6$/ml and T equals total number of samples tested. Manual estimates of motility were conducted using a Makler chamber. At least two Makler chambers were loaded for the measurement to ensure that a representative sample was selected. When the percentage of motile sperm is less than 40 or the percentage of progressive motility is less than 32%, the condition is recorded as asthenospermia. For viability analysis, at least 300 sperm per sample were assessed from eosin-stained preparations. The number of unstained sperm was counted to represent a percentage of viable sperm. For the morphology assessment, two slide smears were prepared from each semen sample. Amorphous head, cytoplasmic droplet, duplicates, and coiled tails were examined to characterize teratozoospermia. Three hundred sperm per slide were evaluated from air-dried Papanicolaou-stained preparations and classified as either normal or abnormal according to the criteria recommended by the WHO.[20]

Detection of DNA fragmentation

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed to detect both single and double DNA strand breaks.[21] In brief, a sperm pellet was obtained from 100 µL semen by centrifuging at 250 g for 5 minutes. The pellet was then resuspended in 1% HAS in phosphate buffer solution (PBS). Then, the sperm was permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate at 4 °C for 2 minutes. A TUNEL mixture (Roche Diagnostic, Manheim, Germany) was deployed onto sperm. Each test included both positive and negative controls to ensure the performance of the assay. Cells for the positive control were treated with 50 µL of DNase solution, while cells for the negative control were not treated with 1%

HAS in PBS. After the cells were incubated for one hour at 37 °C, the sperm cells were observed using an Olympus BX61 fluorescence microscope. Fluorescence detected in sperm was recorded as a positive for DNA fragmentation. At least 300 sperm cells from each sample were accounted for, and the percentage of cells with DNA fragmentation was calculated.

Quantification of 8-oxodGuo adducts in sperm DNA

8-oxodGuo adducts served as a biomarker for oxidative damage, since sperm is vulnerable for oxidative stress damage. Sperm DNA was isolated by using the DTT digestion-based method. The method was slightly modified by procedures recommended by the European Standard Committee on Oxidative DNA Damage to minimize DNA oxidization during DNA isolation procedures.[22] 8-oxodGuo adducts in sperm DNA was determined by a liquid chromatography–-mass spectrometry with an on-line solid-phase extraction (SPE) as reported in recent studies.[23,24] After automatic sample cleanup, DNA samples were injected into an Agilent 1100 series high performance liquid chromatography system interfaced with a PE-SCIEX API 3000 triple quadrupole mass spectrometer with an electrospray ion source. Detection was performed in the positive ion multiple reaction monitoring mode for simultaneous quantification of 8-oxodG and dG. Transitions of the precursors to the product ions were as follows: 8-oxodGuo (m/z 284→168), [$^{15}N_5$]-8-oxodGuo (m/z 289→173), dG (m/z 268→152), and [$^{15}N_5$]-dG (m/z 273→157). With the use of isotopic internal standards and on-line SPE, this method exhibited a low limit of detection (LOD) of 1.8 fmol for 8-oxodGuo, which corresponds to 0.13 adducts/10^6 dG when using 20 μg of DNA per analysis.

Mixture analysis

Levels of metals and semen quality biomarkers below the LOD were replace by LOD/$\sqrt{2}$. For statistical analysis, we included metals with at least 70% of samples having levels above the LOD as continuous variables. Metals with low detection rate (<30%) were excluded from the analysis. Descriptive statistics were calculated for all exposure and outcome variables.

We used BKMR to evaluate both individual and joint effects of the metal mixture, potential non-linear relationships between metals and outcomes of interest, and dose-response trends between metals and outcomes of interest.[18,19] BKMR was fitted using a Gaussian kernel function.[18] Results were obtained based on 10,000 Markov chain Monte Carlo iterations.[19] All BKMR models were adjusted for age, education, smoking status, drinking status, and BMI of the participants. Because exposures in our study are correlated, we conducted the mixture analysis using hierarchical variable selection process in BKMR. The selection process requires grouping of the exposures based on correlations between exposures and similar potential mechanisms of action (e.g., toxic metals vs. essential metals). Thus, we grouped the metal mixtures into two groups: As, Cd, and Pd into group 1 (toxic metals) and Cu, Se, and Zn (essential metals) into group 2.

We estimated the overall mixture effects of the metal mixture (posterior mean estimates and 95% credible intervals) on each semen quality parameter and sperm DNA damage biomarkers. The overall effects were estimated by comparing the biomarker levels when all metals were at a particular percentile (ranging from 25th percentile to 75th percentile) to when all were at their median values. We also evaluated the effect of each individual metal exposure on the outcomes (posterior mean estimates and 95% credible intervals), when all the other metals are fixed at a particular percentile. Additionally, we plotted a dose-response relationship of each metal level on the biomarkers, fixing the other metals at their medians. The metal levels were natural log-transformed for the analyses. All analyses were done in R version 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria) and we used the R package "bkmr" to perform the BMKR analyses.

Table 1. Characteristics of the study participants and outcomes of semen quality and sperm DNA integrity.

Characteristic	Exposure $N = 93$
Age (in years), mean ± SD	48 ± 8
BMI (kg/m^2), mean ± SD	25.2 ± 4.1
Education, %	
Less than high school	48.1
At least high school	51.9
Smoking status, %	
No	46.2
Yes	53.8
Drinking status, %	
No	59.6
Yes	40.4
Work duration (in years)	6.5
Semen quality	
Volume (mL), mean ± SD	1.9 ± 1.0
Level (10^6/mL)	115.6 ± 98.9
Motility (% motile), mean ± SD	55.9 ± 19.2
Viability (%), mean ± SD	73.1 ± 17.6
Morphology (% normal), mean ± SD	15.3 ± 3.0
Amorphous head (%)	83.8 ± 10.1
Oligospermia (%)	2.5
Asthenospermia (%)	28
Teratozoospermia (%)	88.2
Sperm DNA integrity	
8-oxodGuo in sperm (/10^6 dG), mean ± SD	23.4 ± 26.0
DNA fragmentation (%), mean ± SD	38.9 ± 23.8

Results

The characteristics of the 93 participants are shown in Table 1. The participants had an average of 40 years old and had an average BMI of 25.2. About 52% of the participants had at least a high school degree; about 54% were smokers, and about 40% were drinkers, who drink alcohol more than 4 time a week. More than 97% of individuals have healthy sperm count greater than 20×10^6/mL. 18.8% of the subjects had asthenospermia, which percentage of motile sperm <40%. Urinary metals were detected in all samples. The summary statistics (median, 25th percentile, and 75th percentile) of the urinary metal levels of the subjects are shown in Table 2.

Figure 1 shows the associations of the metals, as a mixture, with each outcome of semen quality and sperm DNA integrity. The metals, as a mixture, shows an inverse association with sperm motility, viability, and normal morphology, although the effect estimates are not statistically significant (95% CIs contain the null values). Sperm motility decreases by 0.06 log units (95% CI = (−0.16, 0.04) log units), sperm viability decreases by 0.03 log units (95% CI = (−0.09, 0.03) log units), and sperm normal morphology decreases by 0.10 log units (95% CI = (−0.26, 0.06) log units) when the metal concentrations increase from their median values to their 75th percentiles. Direct association between the metals mixture and semen volume is observed, although the 95% CIs contain the null values. Semen count increases by 0.04 log units (95% CI = (−0.12, 0.20) log units) when metal concentrations increase from their median values to their 75th percentiles. The data mostly suggests null associations of the metal mixture with sperm concentration, 8-oxodGuo, and sperm DNA fragmentation.

The associations of the individual metals, within the mixture, with the outcomes are shown in Figure 2. Although none of the individual associations are statistically significant, Cd has an inverse relationship with sperm motility,

Table 2. Urinary metal levels of the coke-oven workers.

Metal μg/L	Median	25th percentile – 75th percentile
As	141.5	72.7–270.1
Cd	1.0	0.6–1.4
Pb	7.3	3.0–13.6
Cu	16.1	10.0–27.1
Se	17.4	12.8–24.6
Zn	693.8	237.7–1014.9

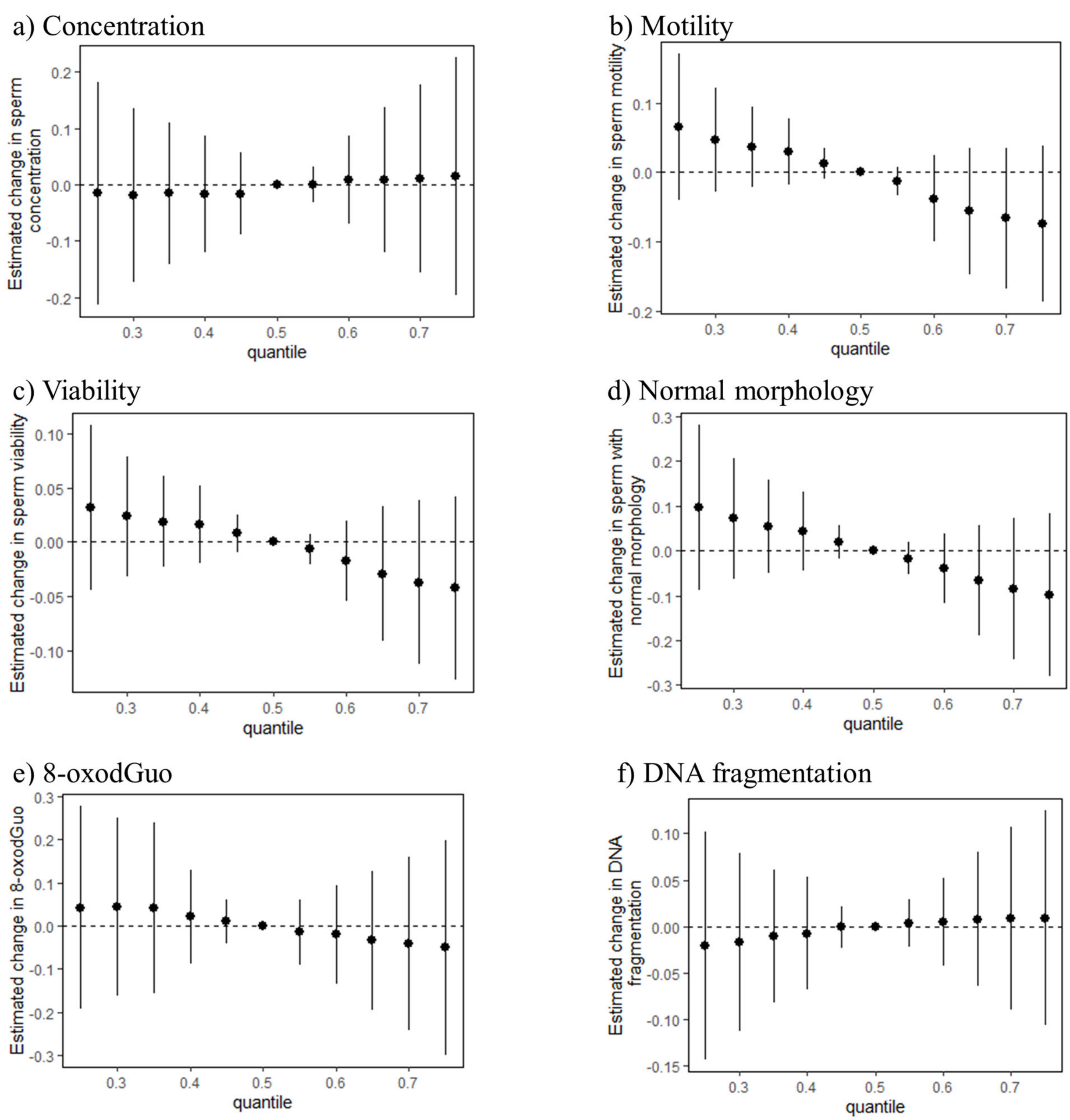

Figure 1. The mixture effects (posterior mean estimates and 95% credible intervals) of the metals on the outcomes on sperm quality, 8-oxodGuo, and sperm DNA fragmentation from BKMR, where the models were adjusted for age, BMI, education, smoking status, drinking status, and job site of the participants.

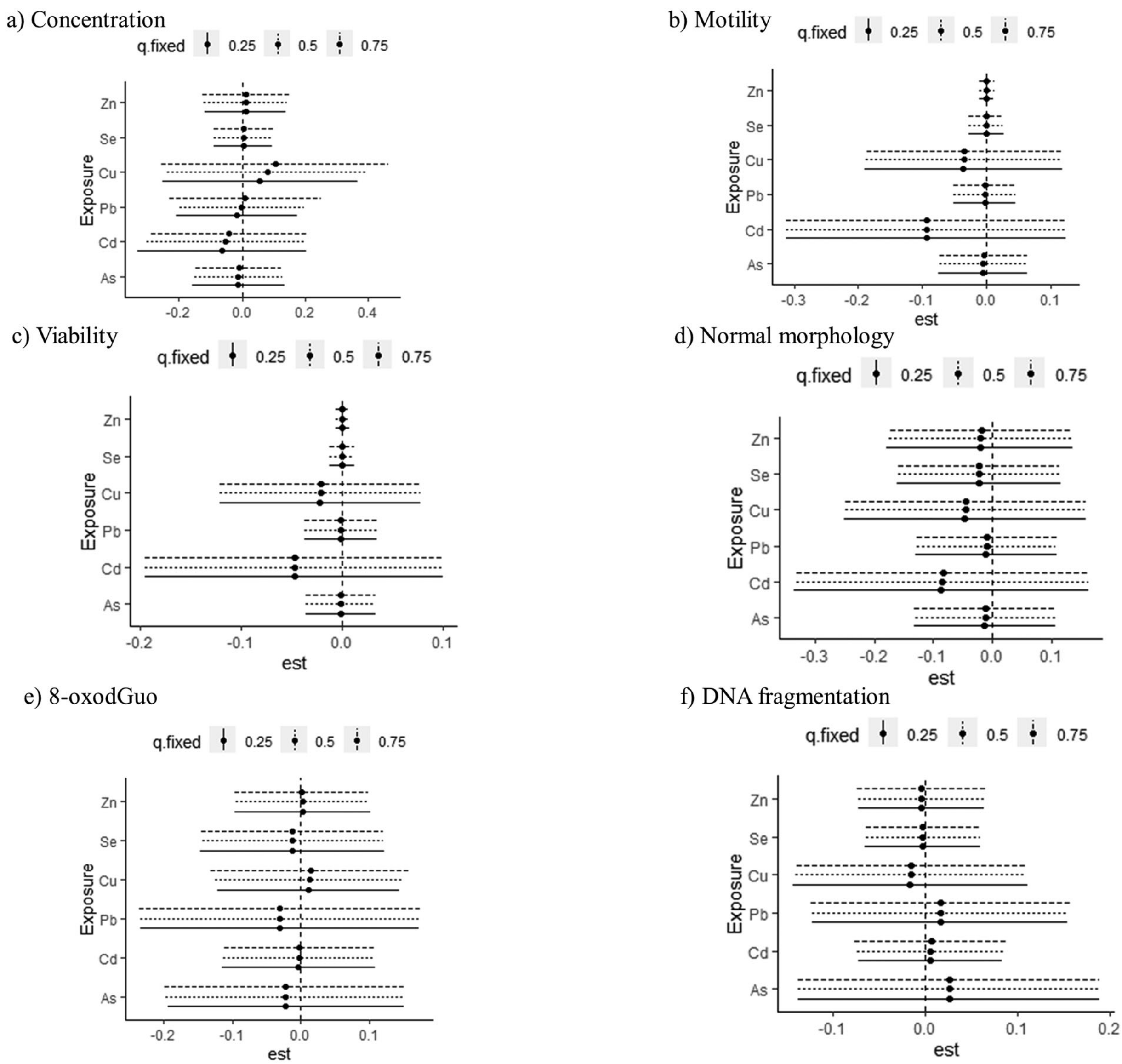

Figure 2. The individual effects (posterior mean estimates and 95% credible intervals) of each metal level, in the mixture, when all other metals are fixed at their 25th, 50th, and 75th percentiles, on sperm quality, 8-oxodGuo, and sperm DNA fragmentation.

viability, and normal morphology. For 8-oxodGuo and DNA fragmentation, there is not much evidence of associations of the metals as a mixture (Fig. 2). The dose response relationship of each metal and the outcomes are shown in Figure 3. Cd shows a non-linear relationship with sperm motility and viability. There is no evidence of any significant interaction effect of an individual metal with other metals in the mixture for any outcome (Fig. 4).

Discussion

Urinary Zn, As, and Se detected in the coke-oven workers were comparable with those in the general Taiwan population, reported in the 2005–2008 Nutrition and Health Survey in Taiwan,[25–27] while urinary Pb (7.3 μg/L) and Cu (16.1 μg/L) in the studied subjects were higher than those (14.5 μg/L, respectively) in the general Taiwan population (5.6 μg/L and 14.5 μg/L, respectively).[25–27] Compared to other occupational workers involved with metals in the process, e.g., welders and smelters, coke-oven workers are exposed to lower concentrations of the metals.[15,28,29]

Although 18% of the workers had asthenospermia, the percentage of asthenospermia (18.7%) of the workers was comparable with that of the general population.[29] We observed a high percentage of teratozoospermia (88%), which was higher than that of the general population (63%).[29] The abnormal morphology largely occurred in sperm heads. That could attribute to declined motility of sperm as 85.4% samples had altered morphology among 18% workers with asthenospermia. Higher numbers of abnormally shaped sperm were reported to be associated with other irregularities of the semen, particularly with motility.[30]

The BKMR analysis revealed an inverse and linear association of the metal mixture with normal morphology, viability, and motility. There was a direct dose-response trend between exposure to the metal mixture and sperm normal morphology, viability, and motility. Given that no

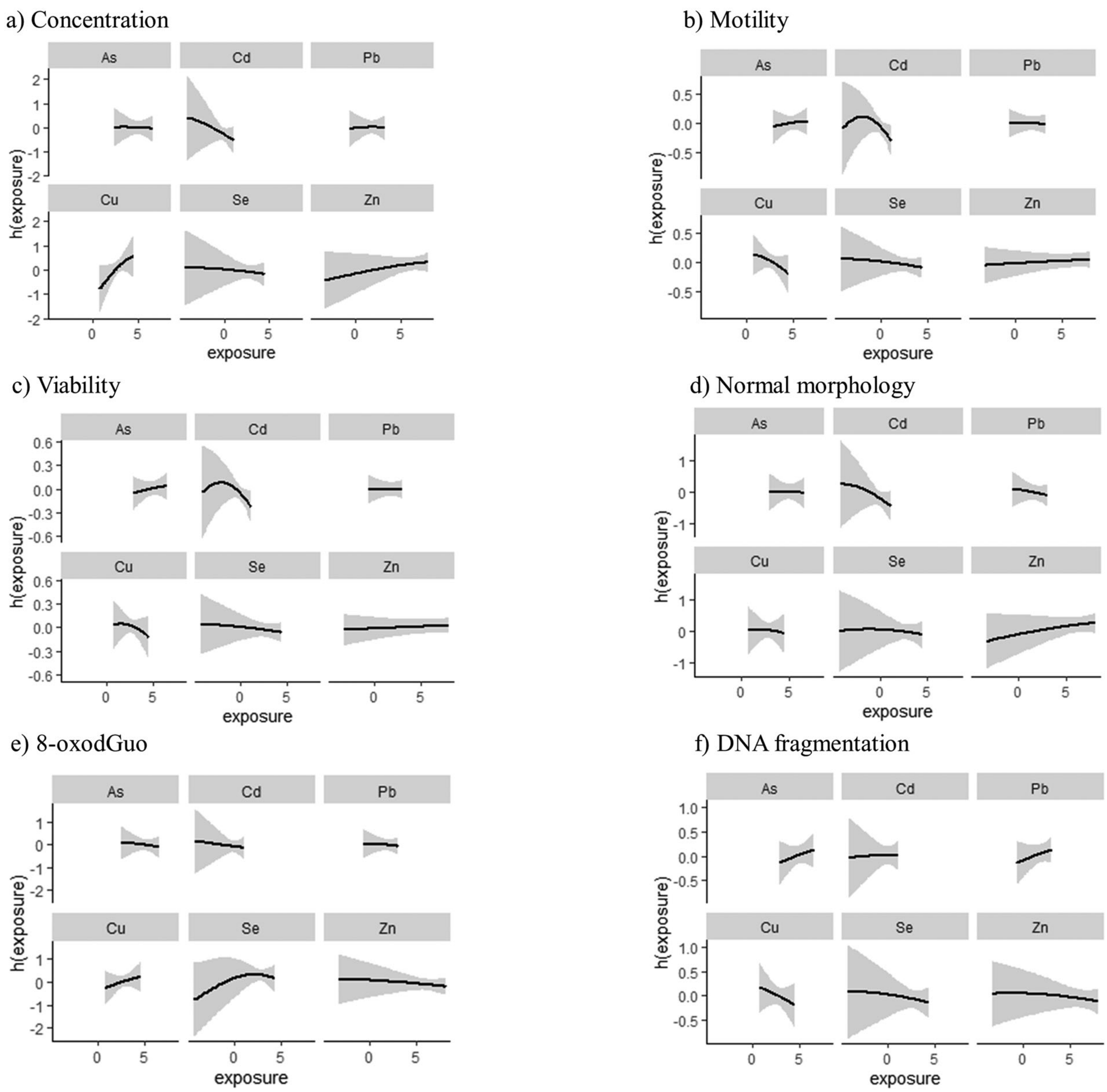

Figure 3. Univariate dose-response relationship with the 95% credible intervals for the effect of a log unit increase in each metal level in urine on log units of sperm quality, 8-oxodGuo, and sperm DNA fragmentation.

interactions among the metals from the BKMR analysis, the metal mixture exhibited additive effects on semen quality, which reflects that the joint effects of the metal mixture were larger than the effects from each individual metal.

Among the metal mixture, Cd was the dominant metal that contributes to the inverse associations with sperm viability, motility, and normal morphology. Other studies also reported similar findings.[31,32] Even low concentrations of Cd exposure were still associated with a moderate, significant change in sperm motility,[12] and sperm morphology.[16] In this study, we found that Cd levels exhibited a U-shaped dose-dependent curve for motility and viability (Figure 3c, d). The inverse association between Cd exposure and sperm motility and viability occurred in the high Cd quantile. That may suggest a potential threshold for the effect of Cd on semen quality,

Multiple mechanisms that mediate Cd reproductive toxicity include structural damage to testis vasculature and blood-testis barrier, inflammation, cytotoxicity on Sertoli and Leydig cells, oxidative stress, and disturbance of the hypothalamus-pituitary-gonadal axis.[33–35] Recent studies reported that Cd influenced semen quality via endocrine disrupting as Cd has potent estrogen- and androgen-like activities *in vivo and in vitro*.[36,37] However, the precise mechanisms underlying the effects of Cd as an endocrine disruptor remain to be elucidated, as studies showed that Cd can significantly reduce semen quality without conclusive evidence of impairment of male reproductive endocrine function or hormone synthesis/regulation.

Cu, Se, and Zn were detected in the urine samples from the coke-oven workers. The average levels of Cu and Zn in the coke-oven workers was comparable with or slightly higher than that in the general population in Taiwan, reported in the 2005–2008 Nutrition and Health Survey in Taiwan.[25–27] Cu, Zn, and Se are essential for reproductive functions within a certain range of levels, but they may be

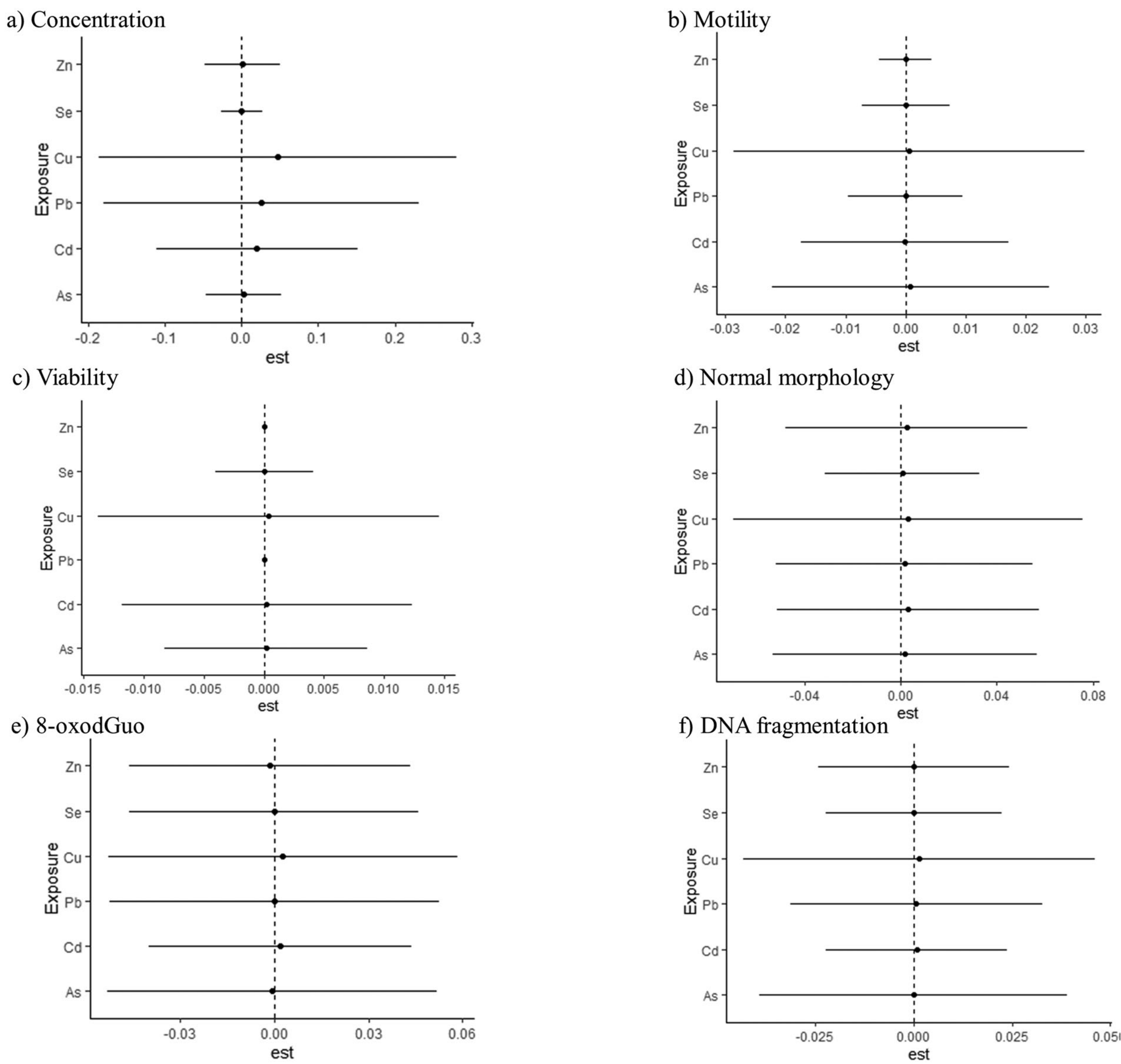

Figure 4. Interactive effect of the metals with 95% credible intervals, obtained by comparing the single metal risk summary when all the other metals are fixed at the 75th percentile vs. when they are fixed at the 25th percentile for outcomes on sperm quality, 8-oxodGuo, and sperm DNA fragmentation, from BKMR models adjusting for age, BMI, education, smoking status, drinking status, and job site of the participants.

harmful beyond that range.[38,39] Only Cu exposure had a positive relationship with sperm concentration. No associations between Zn and Se exposure and semen quality were observed. The Zn and Se detected in this study may exceed the level that is beneficial to semen quality. Other studies reported that Zn and Cu may assist in counteracting the effects in that and other metals.[40,41] For example, Zn was reported to competitively suppress Cd toxic effects by reducing oxidative stress and inflammation.[42] We used BKMR models, adjusted for age, current smoking, alcohol consumption, and BMI, to explore evidence for interaction among metals. However, we found no evidence for interactions involving Cd or Pb with Zn, Cu or Se. Meeker et al. also reported that Cd, Pb, Zn, Cu or Se didn't have interactions in their associations with semen quality.[43]

As stated, oxidative stress and inflammation served as the leading hypothesis of the biological mechanism of reproductive toxicity of metals.[10,42] Contrary to the anticipation, we did not find an association between exposure to individual and joint metals and sperm 8-oxodGuo, which serves as a biomarker of oxidative DNA damage. Future studies with mechanistically based exposure to metal mixtures are recommended to quantify other direct oxidative stress biomarkers, such as reactive oxygen species and malondialdehyde, to gain a better understanding of the effect of metal mixtures on sperm DNA integrity.

Several limitations of the present study should be considered. A limitation was lack of measurement of metals in seminal plasma. A high percentage of seminal plasma samples had Pb and Cd levels < LOD. To achieve the objective of the study including mixture analysis on metal joint effects, we only used urinary metal levels to ensure a suitable sample size to maintain a certain power. The second limitation was the collection of a single urine and semen sample from each subject for measurement of metals and semen quality, respectively. Some metals have long half-lives, such as Pb; a single biological sample may be sufficient. However, some metals are rapidly excreted. Repeated measurements may be required to reflect exposure status in the period of spermatogenesis, which lasts 65–75 days. The reliability of a single semen sample to represent semen quality continues to be debated. Currently, there is limited evidence to confirm whether one semen sample may be adequately representative of semen quality over several weeks in epidemiologic studies. The third limitation was a lack of detailed dietary intake information. The study included basic dietary intake information. Considering high consumption of seafood and rice of the Asian population, more detailed dietary intake data, such as frequency and quantity of seafood and rice consumption, is recommended to be included in future studies and to be treated as a covariate in mixture analysis. The fourth limitation was the small sample size. However, the sample size still sufficed for yielding a statistical power to detect a 1.5-fold difference between the mean concentrations of the outcomes of semen quality and sperm DNA integrity. We recommended a follow up study with a large sample size to confirm the findings of the study.

Conclusion

Our analysis of the metal mixtures with 6 metals had a suggestive relationship with semen quality parameters. The relationship exhibited a dose-dependent fashion. Among the metals, Cd was the primary toxic metal that was inverse associated with sperm motility, viability, and morphology in non-linear fashion. Future larger studies are warranted to confirm the findings and to elucidate mechanisms underlying the link between exposure to metal mixtures and male semen quality parameters. Identifying adjustable occupational risk factors, such as metals, for adverse sperm damage could lead to metal reduction through exposure reduction strategies at the workplace.

Funding

This work was supported by the US National Institute of Environmental Health Sciences (1R15ES018952-01) and the National Institute for Occupational Safety and Health, USA (1R03OH009504-01).

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article. Additional information is available upon reasonable request.

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