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RESEARCH ARTICLE

# Reproductive toxicity of lead, cadmium, and phthalate exposure in men

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**Abstract** Environmental toxicants viz lead or cadmium and phthalate esters (di(2-ethylhexyl) phthalate [DEHP], dibutyl phthalate [DBP], and diethyl phthalate [DEP]) widely found in different environmental strata are linked to deteriorating male reproductive health. The objective was to assess the relationships between the seminal lead, cadmium, and phthalate (DEHP, DBP, DEP) concentrations at environmental level and serum hormone levels and semen quality in non-occupationally exposed men and specify the effect of individual and combined exposure of toxicants on semen quality. A study of 60 male partners of couples attending the Andrology Laboratory of the Reproductive Biology Department, All India Institute of Medical Sciences (AIIMS), New Delhi, India for semen analysis to assess their inability to achieve a pregnancy was selected for the study. The results of univariate and stepwise multiple regression analysis in the unadjusted model showed a significant correlation between lead or cadmium and phthalates DEHP/DBP/DEP and sperm motility, sperm concentration, and DNA damage. After adjusting for potential confounders, an association with lead or DEHP was only observed. The present data shows that lead (Pb) or cadmium (Cd) or phthalates might independently contribute to decline in semen quality and induce DNA damage. Phthalates might influence reproductive hormone testosterone. These findings are significant in light of the fact that men are exposed to a volley of chemicals; however, due to the small sample size, our finding needs to be confirmed in a larger population.

**Keywords** Lead · Cadmium · Phthalate esters · Semen quality · Testosterone · DNA damage

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## Introduction

In recent years, reports on male reproductive health declining for the past few decades have raised concern. Although many factors, e.g., diseases, lifestyle, stress, and obesity, are responsible for declining semen quality, environmental toxicants are also believed to play a vital role in deteriorating male reproductive health. Environmental toxicants viz lead (Pb) or cadmium (Cd) and phthalate esters (di(2-ethylhexyl) phthalate [DEHP], dibutyl phthalate [DBP], and diethyl phthalate [DEP]) widely found in different environmental strata are linked to reduced sperm count and sperm motility (Pant et al. 2008; Jurewicz and Hanke 2011; Jurewicz et al. 2013; Sengupta 2013). Human exposure to metal is through consumption of contaminated food and drinking water, cigarette smoking, and phthalate ester via eating food packed and served in recycled plastic materials. Foods that are highly acidic in nature pose greater risk to human health because plasticizers leach more easily into these foods (Tong et al. 2000; Bernard 2008; Pant et al. 2008).

There is limited documentation of animals and epidemiological studies on the effect of heavy metals (Pb, Cd) and plasticizers (DEHP, DBP, DEP) on semen quality, genotoxicity, and endocrine hormones. Studies investigating the toxicant association with semen quality and sex steroid

hormones in men have been conflicting, finding either no association (Akinloye et al. 2006; Hovatta et al. 1998; Mendiola et al. 2011) or significant association (Pan et al. 2006; Pant et al. 2003; ShuGuang et al. 2011; Telisman et al. 2000, 2007). These chemicals act as an endocrine-disrupting compound by disrupting hormonal regulations, via the hypothalamic-pituitary-testicular axis or direct inhibition of androgen biosynthesis in Leydig cells. While their detrimental effects are known, the mechanism of action is not known (Iavicoli et al. 2009; Siu et al. 2009; Vigeh et al. 2011; Jurewicz and Hanke 2011; Jurewicz et al. 2013).

Regarding genotoxicity studies of Pb and Cd, in vitro studies using the alkaline comet assay found that these heavy metals induced DNA damage in fibroblast cells (Mouron et al. 2001), lysosomes (Fotakis et al. 2005), lymphocytes (Biasiak 2001), and sperm (Anderson et al. 1997). Epidemiological studies showed pottery-glaze ceramic workers; storage battery workers; secondary Pb recovery unit workers; and welders of India (Danadevi et al. 2003; Grover et al. 2010), China (Zhijian et al. 2006), Poland (Palus et al. 2003), and Croatia (Kašuba et al. 2012) having induced DNA damage in peripheral lymphocytes as detected by the comet assay. However, another investigator showed that the sperm chromatin structure was not related to blood lead concentration, but some indication of deterioration of sperm chromatin was found in men with the highest concentration of lead within spermatozoa (Bonde et al. 2002). However, data in non-occupational setting of humans is lacking. Regarding phthalates, US studies reported an association between the urinary phthalate metabolite level and sperm DNA damage using the neutral comet assay (Duty et al. 2003; Hauser et al. 2007; Jurewicz et al. 2013). However, a Swedish study failed to establish any association between phthalate monoesters and sperm DNA damage determined by the sperm chromatin structure assay (Jönsson et al. 2005). In vitro studies using the alkaline comet assay (single-cell gel electrophoresis) found di-*n*-butyl phthalate (DBP) and di-isobutyl phthalate (DiBP), DEHP, and MEHP to be genotoxic in human leukocytes (Anderson et al. 1999) and mucosal cells of the upper aerodigestive tract (Kleinsasser et al. 2000a, b). Thus, the literature survey indicates that despite widespread general population exposure to these xenobiotics, sparingly and conflicting epidemiological data exist on the effects of toxicant exposure to male reproductive parameters.

To our knowledge, no human studies have investigated the association with the specific and combined exposure of metals and phthalate esters on semen quality, genotoxicity, and serum hormone levels. The general population is simultaneously exposed to a cocktail of chemicals that might act as endocrine disruptors; the objective was to assess the relationships between the seminal lead, cadmium, and phthalate (DEHP, DBP, DEP) concentrations at environmental level and serum hormone levels and semen quality in non-occupationally exposed men and specify the effect of individual and combined exposure of toxicants on semen quality.

## Materials and methods

### Subject selection and semen analysis

Male partners of couples age 21–40 years old attending the Andrology Laboratory of the Reproductive Biology Department, All India Institute of Medical Sciences (AIIMS), New Delhi, India for semen analysis to assess their inability to achieve a pregnancy were selected. Men recruited to the study were from New Delhi and its surrounding areas. The proposed study was approved by the Institutional Ethical Committee. Subjects occupationally exposed to metals or with past medical history (mainly testicular dysfunction/history of urogenital abnormality/mumps, tuberculosis, or surgical operation; using drugs known to affect gonadal function) were excluded from the study. Informed consent was obtained from each participant prior to the study. The volunteers were given an option to withdraw from the study at any time. The participation rate of the volunteers was noted. Sixty men agreed to participate in the study while 31 men failed in the predetermined selection criteria. Semen of volunteers was collected by masturbation into a sterile wide mouth glass container after at least 3–5 days of sexual abstinence. Abstinence period and spillage, if any, were recorded. Semen analysis was carried out following the protocols of the WHO (2010). The liquefaction time, pH, odor, viscosity, volume, presence of pus or epithelial cells, sperm agglutination, sperm motility, and sperm concentration were noted. Sperm morphology was determined according to Kruger's strict criteria (WHO 2010). Simultaneously, a detailed questionnaire was filled, which included age, education, social status, occupation, diet, smoking, tobacco chewing habit, and medical and surgical history.

### Metal analysis

ICP Multi-element standard solution IV purchased from Merck Chemicals, Germany (product no. 1.11355.0100, batch no. HC061563, dated March 7, 2010) was used as reference standard. Suprapure nitric acid and hydrogen peroxide were purchased from Merck Chemicals, India.

Approximately 1 ml of semen was digested with nitric acid (65 %) and hydrogen peroxide (30 %) ($HNO_3$:$H_2O_2$, 5:1) in a microwave. The digested samples were cooled, diluted up to 5 ml with deionized water, and analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES JY 2000). The precision for lead and cadmium was calculated to be 1.41 and 0.32, while the limit of detection was 1.9 and 0.28 ppb, respectively. A sample blank was prepared with

each set of samples to control for possible metal contamination from external sources.

### Instrumental analysis

Phthalate esters, mainly diethyl phthalate (DEP), dibutyl phthalate (DBP), and diethyl hexyl phthalate (DEHP), were detected and quantified using an Ultra Pressure Liquid Chromatography (UPLC) Acquity System (Waters) coupled with a mass spectrometer (API 4000), and data were acquired with Analyst 1.6 software (AB Sciex).

### Conditions of the instrument

Separation of phthalate esters was achieved on Acquity UPLC VHE C18, 1.7 μm, 2.1×50-mm column at 22 °C temperature with the eluting solvent methanol–water (70:30); run time is 5.0 min at a flow rate of 0.3 ml per min in an isocratic condition, and pressure was maintained at 3,500 psi. Positive multiple reaction monitoring (MRM) method for phthalates was developed on the basis of these source parameters: CAD-10.00, Curtain gas-32.00, GS1-10.00, GS2-0.00, IS-5500.00, and temperature-150.00.

Compound parameters are as follows:

| | Compound parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| | Q1 mass (Da) | Q3 mass (Da) | Dwell (msec) | DP | EP | CE | CXP |
| DEP | 223.100 | 177.000 | 200.00 | 63.00 | 5.00 | 12.00 | 13.00 |
| DBP | 279.200 | 205.200 | 200.00 | 100.00 | 10.00 | 9.00 | 13.00 |
| DEHP | 391.200 | 167.000 | 200.00 | 88.00 | 12.00 | 13.00 | 11.00 |

### Extraction of phthalates

Briefly, 1 ml of methanol (HPLC grade) was added to the aliquots of semen, extracted with a mixture of hexane:ethyl ether (1:1), agitated, and centrifuged at 2,000×*g* for 5 min. The organic phase was collected, concentrated, and dried completely under nitrogen, and the sample was redissolved in 1.0 ml methanol, filtered through 0.22-μm SS syringe filter. Ten microliters of the sample was injected into MASS through Acquity UPLC autosampler. Reagent blanks and spiked sample were also run along with unknown samples. The quantification was performed on the basis of count per second compared to the standard chromatogram. Standards of DEP (99.9 %), DBP (99.9 %), and DEHP (99.7 %) were procured from Sigma-Aldrich, USA. Limit of detection (LOD) is 1.0 ppb and limit of quantitation (LOQ) is 3.5 ppb for all phthalates. Recovery was found to be 92, 85, and 82 % for DEP, DBP, and DEHP, respectively.

### Hormone measurements

Approximately 3 ml of blood was drawn from the subjects during a morning clinic visit after 8 h of fasting. After separating the serum, FSH, LH, and testosterone were determined by the Architect reagent kit specifically for each hormone. The assay is a chemiluminescence microparticle immumoassay for the quantitative determination of specific hormones on the Architect Abbott i1000SR system.

### Comet assay

Briefly, 10 μl sperm and low-melting point agarose (Sigma-Aldrich) were gently added to a fully frosted slide covered with normal agarose. Then, the slides were submerged in a cold, freshly prepared lysis buffer solution (2.5 M sodium chloride [NaCl], 100 mM EDTA, 10 mM Tris (hydroxymethyl) aminomethane hydrochloride, 10 % dimethylsulfoxide [DMSO], and 1 % Triton X-100, pH 10.0) for 1 h and incubated overnight at 37 °C with 100 μg/ml proteinase K in 2.5 M NaCl, 100 mM EDTA, and 10 % DMSO (pH 7.40). The slides were then transferred to fresh electrophoretic buffer for 20 min (300 mM sodium hydroxide, 1 mM EDTA; pH 12.0), washed twice with neutralization buffer, and stained with 20 μg/ml ethidium bromide. The slides were observed under a fluorescence microscope (Nikon, Towa Optics, India). For each sample, two duplicate slides were prepared, and a total of 100 cells were scored. The percentage of tail DNA, tail length, and tail moment (TM) was evaluated by the CometScore software image analysis system.

### Statistical analysis

The data were analyzed by Stata 11.1 (Stata Corps., TX, USA) and expressed as mean (SD) and frequency (percentage). Many variables were not following the normal distribution after transformation, we applied stepwise multiple regression analysis taking these toxicants lead, cadmium, and phthalates in original form variables because they are not fully known as predictor of fertility or semen parameters and they are not highly correlated among themselves to be considered as collinear variable. Other covariates - age, BMI, diet, smoking, and tobacco chewing and alcohol consumption were not found statistically related with outcomes in this study. Non normality condition of variables is short coming of this study. Univariate and stepwise multiple regression analysis was carried out to see the independent associated risk factors contributing to infertility. *P* value $<0.05$ was considered statistically significant.

**Table 1** Demographic characteristics of non-occupationally exposed subjects

| | ($N$=60) |
|---|---|
| Age years (mean±SD) | 31.81±5.27 |
| BMI (kg/m$^2$) | 24.04±3.55 |
| Diet | |
| Vegetarian | 29 (48.33 %) |
| Non-vegetarian | 31 (51.67 %) |
| Smoking | |
| Yes | 7 (11.67 %) |
| No | 53 (88.33 %) |
| Tobacco chewing | |
| Yes | 14 (23.33 %) |
| No | 46 (76.67 %) |
| Alcohol | |
| Yes | 19 (31.67 %) |
| No | 41(68.33 %) |

## Results

Table 1 shows that the participants' average age was 31.81± 5.27 years, with a mean BMI of 24.04 kg/m$^2$. Most men were non-smokers (i.e., 88.33 %) while 11.67 % were smokers; non-tobacco chewers comprised 76.67 % of the subjects, while tobacco chewers 23.33 %. Non-alcoholic subjects were 68.33 % and alcoholics 31.67 %. Of the participants, 48.33 % were vegetarians and 51.67 % were non-vegetarians.

Table 2 depicts semen characteristics and comet and reproductive endocrine function parameters. Of the 60 men selected for the study, the liquefaction time varied between 15 and 20 min and mean volume was 3.04 ml. Regarding sperm concentration, 25 men (41.67 %) had a sperm concentration $<15\times10^6$/ml and 35 men (58.33 %) had a sperm concentration $>15\times10^6$/ml, while sperm motility of 37 men (61.67 %) was <40 % and that of 23 subjects (38.33 %) was >40 %. The mean (SD) sperm concentration was 22.6±15.6 millions/ml; motility was 34.8±22 %, and normal morphology was 32.6± 10.37 %. The mean (SD) concentrations of Pb and Cd were 6.18±2.16 and 4.91±2.12 μg/dl, respectively, while those of phthalates DBP, DEHP, and DEP were 0.97±0.55, 0.59± 0.37, and 0.90±0.57 μg/ml, respectively. The comet tail length was 33.43±28.44 μm, percentage of DNA in the tail 14.78.18±10.47, and TM 12.11±8.83. The mean (SD) concentration of testosterone was 4.95±1.21 ng/ml, FSH 5.32± 1.549 mIU/ml, and LH 5.21±1.9 mIU/ml.

Table 3 shows the univariate and stepwise multiple regression analysis in subjects when reproductive parameters were each considered with respect to the following variables: toxicants (lead, cadmium, DEP, DBP, DEHP), age, body mass index, tobacco, smoking, alcohol, and diet. In the unadjusted model, a significant correlation between lead ($\beta$=−3.33, $P$<0.01; $\beta$=−2.71, $P$<0.003) or cadmium ($\beta$=−3.38, $P$<0.01; $\beta$=−2.80, $P$<0.003) or phthalates DEHP ($\beta$=−25.06, −20.61 $P$<0.001), DBP ($\beta$=−10.05, $P$<0.05; $\beta$=−6.42, $P$<0.05), and DEP ($\beta$=−11.55, $\beta$=−5.55, $P$<0.01) and sperm motility and sperm concentration, respectively, was observed. However, in the adjusted model, a significant association was observed with Pb ($\beta$=−2.43, $P$<0.05; $\beta$=−1.97, $P$<0.02) and DEHP ($\beta$=−21.63, $P$<0.004; $\beta$=−17.83, $P$<0.001) with sperm motility and sperm concentration. There was an association between normal morphology of sperm and lead ($\beta$=−1.27, $P$<0.04) or cadmium ($\beta$=−1.16, $P$<0.06) and DEHP ($\beta$=−10.35, $P$<0.004) in the unadjusted model. An inverse association was observed with DEHP ($r$=−10.35, $P$<0.001) with normal morphology of sperm in the adjusted model.

With respect to the comet assay parameters, the correlation coefficient with seminal lead was $\beta$=4.58 ($P$<0.006) for tail length; $\beta$=1.74, $P$<0.005 for Tail% and $\beta$=1.53, $P$<0.003 for TM. A positive correlation was observed between cadmium and tail length $\beta$=3.77, $P$<0.02; Tail% $\beta$=1.62 $P$<0.01; and TM $\beta$=1.29, $P$<0.02. With respect to phthalates, a

**Table 2** Semen analysis characteristics, comet assay parameters, plasma hormone levels, and toxicant levels in non-occupationally exposed subjects

| Semen parameters | Mean (SD) | $n$ (%) |
|---|---|---|
| Sperm concentration (10$^6$/ml) | 22.6 (15.6) | |
| Participants with sperm concentration <15 (10$^6$/ml) | | 25 (41.67) |
| Participants with sperm concentration >15 (10$^6$/ml) | | 35 (58.33) |
| Total sperm motility (%) | 34.8 (22) | |
| Participants with sperm motility <40 % | | 37 (61.67) |
| Participants with sperm motility >40 % | | 23 (38.33) |
| Normal sperm morphology (%) | 32.6 (10.7) | |
| Comet assay parameters | | |
| Tail length (μm) | 33.43 (28.44) | |
| %DNA in tail | 14.78 (10.47) | |
| Tail moment | 12.11 (8.83) | |
| Hormone parameters | | |
| Testosterone (ng/ml) | 4.95 (1.21) | |
| FSH (mIU/ml) | 5.32 (1.54) | |
| LH (mIU/ml) | 5.21 (1.92) | |
| Toxicant levels | | |
| Metals | | |
| Pb (μg/dl) | 6.18 (2.16) | |
| Cd (μg/dl) | 4.91 ( 2.12) | |
| Phthalates | | |
| DEP (μg/ml) | 0.90 (0.57) | |
| DBP (μg/ml) | 0.97 ( 0.55) | |
| DEHP (μg/ml) | 0.59 ( 0.37) | |

**Table 3** Univariate and stepwise multiple regression analysis of 60 subjects for associations between semen quality parameters, comet parameters, and potential confounders: toxicants (lead, cadmium, DEP, DBP, DEHP), age, body mass index, tobacco, smoking, alcohol, and diet

| | Unadjusted | | | Adjusted | | |
|---|---|---|---|---|---|---|
| Variables | Regression coefficient ($\beta$) | (95 % CI) | *P* | Regression coefficient ($\beta$) | (95 % CI) | *P* |
| Sperm motility (%) | | | | | | |
| Lead | −3.33 | −5.85, −0.80 | <0.01 | −2.43 | −4.87, −0.001 | <0.05 |
| Cadmium | −3.38 | −5.91, −0.75 | <0.01 | | | |
| DEP | −11.55 | −21.06, −2.04 | <0.01 | | | |
| DBP | −10.05 | −20.22, −0.12 | <0.05 | | | |
| DEHP | −25.06 | −39.21, −10.91 | <0.001 | −21.63 | −35.85, −7.41 | <0.004 |
| Age | −0.21 | −1.30, 0.89 | <0.71 | | | |
| Body mass index | −0.30 | −1.93, 1.32 | <0.71 | | | |
| Tobacco | 1.73 | −11.84, 15.28 | <0.80 | | | |
| Smoking | −2.95 | −20.81, 14.91 | <0.74 | | | |
| Alcoholic | −0.41 | −12.74, 11.93 | <0.94 | | | |
| Diet | −6.49 | −17.85, 4.86 | <0.25 | | | |
| Sperm concentration ($10^6$)/ml | | | | | | |
| Lead | −2.71 | −4.46, −0.94 | <0.003 | −1.97 | −3.16, −0.33 | <0.02 |
| Cadmium | −2.80 | −4.59, −1.02 | <0.003 | | | |
| DEP | −5.55 | −12.48, −1.38 | <0.01 | | | |
| DBP | −6.42 | −13.69, −0.84 | <0.05 | | | |
| DEHP | −20.61 | −30.27, −10.93 | <0.001 | −17.83 | −27.41, −8.24 | <0.001 |
| Age | −0.15 | −0.94, 0.62 | <0.68 | | | |
| Body mass index | −0.21 | −1.36, 0.94 | <0.71 | | | |
| Tobacco | 1.02 | −8.61, 10.65 | <0.83 | | | |
| Smoking | −2.79 | −15.47, 9.88 | <0.66 | | | |
| Alcoholic | −4.36 | −13.05, 4.32 | <0.32 | | | |
| Diet | −6.30 | −14.29, 1.68 | <0.12 | | | |
| Normal morphology | | | | | | |
| Lead | −1.27 | −2.48, −0.05 | <0.04 | | | |
| Cadmium | −1.16 | −2.41, 0.08 | <0.06 | | | |
| DEP | −5.80 | −10.52, −1.36 | <0.01 | | | |
| DBP | −3.96 | −8.79, 0.87 | <0.10 | | | |
| DEHP | −10.35 | −17.18, −3.52 | <0.004 | −10.35 | −17.18, −3.53 | <0.001 |
| Age | −0.09 | −0.60, 0.42 | <0.72 | | | |
| Body mass index | 0.15 | −0.61, 0.91 | <0.69 | | | |
| Tobacco | 2.47 | −3.87, 8.83 | <0.43 | | | |
| Smoking | 4.33 | −4.00, 12.67 | <3.03 | | | |
| Alcoholic | 3.51 | −2.24, 9.24 | <0.23 | | | |
| Diet | −3.91 | −9.22, 1.39 | <0.14 | | | |
| Tail length | | | | | | |
| Lead | 4.58 | 1.35, 7.82 | <0.006 | 3.79 | 0.56, 7.02 | <0.02 |
| Cadmium | 3.77 | 0.40, 7.15 | <0.02 | | | |
| DEP | 6.86 | −5.90, 19.62 | <0.28 | | | |
| DBP | 12.45 | −0.71, 25.6 | <0.05 | | | |
| DEHP | 24.64 | 5.56, 43.73 | <0.01 | 11.72 | 0.67, 22.77 | <0.03 |
| Age | 0.25 | −1.16, 1.67 | <0.72 | | | |
| Body mass index | 0.31 | −1.78, 2.42 | <0.76 | | | |
| Tobacco | −7.01 | −24.43, 10.42 | <0.42 | | | |
| Smoking | −5.47 | −28.52, 17.57 | <0.63 | | | |

**Table 3** (continued)

| Variables | Unadjusted | | | Adjusted | | |
|---|---|---|---|---|---|---|
| | Regression coefficient ($\beta$) | (95 % CI) | $P$ | Regression coefficient ($\beta$) | (95 % CI) | $P$ |
| Alcoholic | 7.61 | −8.20, 23.42 | <0.34 | | | |
| Diet | 5.82 | −8.94, 20.57 | <0.43 | | | |
| %DNA in tail | | | | | | |
| Lead | 1.74 | 0.55, 2.97 | <0.005 | 1.31 | 0.172, 3.74 | <0.02 |
| Cadmium | 1.62 | 0.39, 2.84 | <0.01 | | | |
| DEP | 3.80 | −0.84, 8.44 | <0.11 | | | |
| DBP | 4.63 | −0.21, 9.48 | <0.05 | | | |
| DEHP | 12.25 | 5.55, 18.94 | <0.001 | 10.39 | 3.74, 17.05 | <0.003 |
| Age | 0.06 | −0.46, 0.57 | <0.86 | | | |
| Body mass index | −0.15 | 0.92, 0.62 | <0.69 | | | |
| Tobacco | −1.98 | −8.42, 4.45 | <0.53 | | | |
| Smoking | −2.61 | −11.09, 5.86 | <0.53 | | | |
| Alcoholic | 0.76 | −5.10, 6.63 | <0.79 | | | |
| Diet | 1.77 | −3.66, 7.22 | <0.52 | | | |
| Tail moment | | | | | | |
| Lead | 1.53 | 0.54, 2.52 | <0.003 | 1.20 | 0.23, 2.16 | <0.01 |
| Cadmium | 1.29 | 0.26, 2.34 | <0.02 | | | |
| DEP | 6.97 | 0.71, 14.66 | <0.10 | | | |
| DBP | 2.40 | −1.76, 6.57 | <0.25 | | | |
| DEHP | 0.41 | 4.11, 15.51 | <0.001 | 8.13 | 2.49, 13.75 | <0.005 |
| Age | 0.09 | −0.34, 0.53 | <0.67 | | | |
| Body mass index | −0.06 | −0.71, 0.58 | <0.83 | | | |
| Tobacco | −2.58 | −7.98, 2.81 | <0.34 | | | |
| Smoking | −2.09 | −7.98, 2.81 | <0.56 | | | |
| Alcoholic | 1.16 | −3.78, 6.10 | <0.64 | | | |
| Diet | 3.00 | −1.53, 7.54 | <0.19 | | | |
| Testosterone | | | | | | |
| Lead | 0.04 | −0.11, 0.18 | <0.58 | | | |
| Cadmium | 0.01 | −0.13, 0.16 | <0.87 | | | |
| DEP | 0.70 | −0.36, 1.77 | <0.19 | | | |
| DBP | −0.64 | −1.20, −0.09 | <0.02 | −0.61 | −1.20, −0.02 | <0.04 |
| DEHP | −1.06 | −1.87, −0.24 | <0.02 | −0.96 | −1.82, −0.11 | <0.02 |
| Age | 0.07 | −0.87, 0.33 | <0.37 | | | |
| Body mass index | −0.13 | −0.10, 0.07 | <0.76 | | | |
| Tobacco | −0.26 | −0.48, 1.00 | <0.49 | | | |
| Smoking | −0.77 | −1.73, 0.19 | <0.11 | | | |
| Alcoholic | −0.54 | −1.21, 0.12 | <0.10 | | | |
| Diet | 0.18 | −0.44, 0.81 | <0.56 | | | |

correlation between mean DBP and tail length ($\beta$=12.45, $P$<0.05) and tail percentage of DNA (%DNA) ($\beta$=4.63, $P$<0.05) was observed. There was a significant positive correlation between mean DEHP and tail length ($\beta$=24.64, $P$<0.01), tail %DNA ($\beta$=12. 25, $P$<0.001), and TM ($\beta$= 0.41, $P$<0.001). In the adjusted stepwise multiple regression analyses, the independent association between tail length and Pb or DEHP was $\beta$=3.79, $P$<0.02; $\beta$=11.72, $P$<0.03; %DNA in the tail was $\beta$=1.31, $P$<0.02; $\beta$=10.39, $P$<0.003; and tail moment $\beta$=1.20, $P$<0.01; $\beta$=8.13, $P$<0.005. No such correlation was established with DEP. There was no significant association between lead or cadmium and endocrine hormones viz testosterone, FSH, and LH. However, phthalates DBP/DEHP showed a negative association with testosterone. DEHP/DBP was not significantly associated with FSH and LH but the trend of regression coefficient was negative. Age,

body mass index, tobacco, smoking, alcohol, and diet were not associated with any of the reproductive or comet assay parameters. The synergistic effect of toxicants on semen quality parameters was not observed.

## Discussion

The main outcome of the study is that environmental exposure to lead, cadmium, and phthalates DBP, DEHP, or DEP independently might decrease sperm concentration and motility or induce DNA damage by the comet assay. The synergistic effect of phthalates and metals studied on deteriorating semen quality was not observed. Besides, phthalates might influence reproductive hormones; however, no evidence of association between seminal heavy metal concentration (lead, cadmium) and circulating levels of hormones testosterone, LH, and FSH was found. This is consistent with a cross-sectional, case-control, and prospective double-blind study of Nigerians (Akinloye et al. 2006) and Spanish (Mendiola et al. 2011), where seminal Cd levels of 65–165 or 0.09 μg/dl and seminal lead concentration of 3.0 μg/dl had no significant influence on endocrine hormones. Similarly, urinary cadmium level was also not associated with sex steroid hormone concentration in a large, nationally representative sample of US men (Menke et al. 2008). On the contrary, other studies showed significant effect of blood/urinary lead or cadmium levels on sex steroid hormones in environmentally or occupationally exposed Chinese (Zeng et al. 2002, 2004), Italian (Ciarrocca et al. 2013), Croatian (Telisman et al. 2000; 2007; Jurasovic et al. 2004), and US men (Meeker et al. 2010). Thus, studies investigating the association have been inconsistent, finding either no association or a positive association. The discrepancy might be attributed to different concentrations of heavy metal in three body fluids (semen, blood, and urine) and duration of metal exposure or to inadequate control of confounders that are also associated with higher testosterone levels in men. The lack of association with serum FSH, LH, and testosterone implies that the lead might directly affect testicular function. Regarding phthalates, the current investigation showed an inverse association of phthalates DEHP and DBP with testosterone; however, no association with FSH or LH could be established. This is in agreement with a Chinese study where reduction of serum testosterone was associated with higher levels of urinary and seminal phthalates and its metabolites in occupationally and environmentally exposed men (Pan et al. 2006; ShuGuang et al. 2011). Although in the Swedish study no associations were found between phthalate metabolites and reproductive hormones (Jönsson et al. 2005), however, in the US study, associations between phthalate metabolite urinary concentrations and altered levels of inhibin B and FSH were found (Duty et al. 2005). The exact mechanism by which a significant decrease in the testosterone level and non-significant decreases of LH and FSH levels occur remains to be elucidated, but in vitro and in vivo studies suggest that phthalates might suppress testosterone biosynthesis by influencing genes involved in cholesterol transport and testosterone synthesis or influence the normal feedback regulation of the HPT axis (Fabjan et al. 2006).

The lead- and cadmium-related effects on semen quality have also been reported by other investigators. Studies by Mendiola et al. (2011), Hernandez-Ochoa et al. (2005), and Benoff et al. (2009) reported that lead (0.2–2.9 μg/dl) and cadmium concentration (0.028 or 0.08 μg/dl) in seminal fluid was associated with one or more seminal parameters and increase in sperm chromatin condensation. On the contrary, Hovatta et al. (1998), Keck et al. (1995), and Xu et al. (1993) indicated that no correlation was found between seminal cadmium/lead level and sperm parameters. Regarding phthalates, in the Chinese study (Zhang et al. 2006; ShuGuang et al. 2011), the mean concentrations of DEP (0.13–1.32 μg/ml), DBP (0.09–0.57 μg/ml), and DEHP (0.08–0.98 μg/ml) was low as compared to the current investigation. The high value of the toxicants observed in the present study as compared to those reported by others might be due to variations in exposures, heterogeneity of the population, and different analytical methods of detection.

As reported in our earlier studies, we also found inverse relationships between heavy metals/phthalates and sperm concentration and sperm motility. These toxicants are strongly associated with mitochondrial dysfunction, free radical production, or lipid peroxidation, and these parameters likely represent the interrelated aspects of the overall status of sperm. A significant ROS production causes depletion in the antioxidant levels and enhances lipid peroxidation, which might finally lead to inhibition of sperm function. Damage to the sperm membrane reduces sperm motility. Leydig cells also can influence secretion of testosterone, which affects Sertoli cell function and spermatogenesis (Pant et al. 2008; Pizent et al. 2012; Fotakis et al. 2005; Fabjan et al. 2006).

Lead, cadmium, and phthalates (DEHP, DBP) induce DNA damage. This is in agreement with the results studies of lead/cadmium in occupationally exposed Indian, Croatian, and Chinese workers recruited in the recycling and manufacture of automotive batteries, secondary lead recovery units, and welding units of small-scale industries. Data on genotoxicity of occupational Pb exposure in humans using comet assay show significant evidence of association (Kašuba et al. 2012; Palus et al. 2003; Zhijian et al. 2006; Danadevi et al. 2003; Grover et al. 2010). In vitro studies found these toxicants to be associated with DNA damage using lymphocytes (Anderson et al. 1997, 1999), human lung fibroblast cell lines (Mouron et al. 2001), and mucosal cells of the upper aerodigestive tract (Kleinsasser et al. 2000a, b, 2001). In the US population, sperm DNA damage was associated with urinary MEP and MEHP at environmental levels (Duty et al.

2003; Hauser et al. 2007; Jurewicz et al. 2013). Another study demonstrated a link between DEHP concentration in ambient air and the adverse effects on sperm motility and chromatin and DNA integrity (Huang et al. 2011). Sperm DNA damage does not influence the sperm count or morphology but is associated with incidences of miscarriages and birth defects in the offspring. The mechanism underlying the genotoxicity might involve metal/phthalate-induced production of ROS. The high ROS production concomitant with mitochondrial dysfunction results in oxidative stress inhibition of antioxidant enzymes, which finally leads to induction of DNA strand beaks (Grover et al. 2010; Fotakis et al. 2005; Pant et al. 2008). The present study is consistent with the animal studies that DEHP can cause obvious toxic effects on reproductive function in male rats than DBP (Kwack et al. 2009; Mei et al. 2009).

The present data shows that Pb or Cd or phthalates DEHP, DEP, and DBP might independently contribute to decline in semen quality and induce DNA damage. Phthalates might influence the reproductive hormone testosterone. The synergistic effects of heavy metal burdens lead and cadmium or phthalates DEHP and DBP on declining semen quality was not observed. These findings are significant in light of the fact that men are exposed to a volley of chemicals However, due to the small sample size, our finding needs to be confirmed in a larger population.

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