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Genomic instability in adult men involved in processing electronic waste in Northern China

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ABSTRACT

Background: Managing and recycling electronic waste (e-waste), while useful and necessary, has resulted in significant contamination of several environments in China. The area around Tianjin, China has become one of the world's largest e-waste disposal centers, where electronics are processed by manually disassembly or burning, which can result in serious exposure of workers to a multitude of toxicants.
Objective: The present study assessed potential genomic damage in workers involved in recycling e-waste.
Methods: To detect cytogenetic and DNA damage, chromosomal aberrations (CA), cytokinesis blocking micronucleus (CBMN) and the comet assay were performed. Concentrations of some trace elements, markers of oxidative stress and polychlorinated biphenyls (PCBs) in whole blood or serum were measured, and relationships among the markers described above, age, and duration of exposure were analyzed. The profiles of expression of genes in lymphocytes in peripheral blood were assessed to determine the status of the regulation of genes involved in genome stability.
Results: Concentrations of 28 PCB congeners in the whole blood of the exposed group were significantly ($P < 0.001$) greater than those in the control individuals. Frequency of CA (8.01%) and CBMN (26.3‰) in lymphocytes and the level of DNA damage in the lymphocytes and spermatozoa of the exposed men were also significantly ($P < 0.0001$) greater than those of the controls. There were significant relationships between CA, CBMN, DNA damage and duration of exposure. Concentrations of malondialdehyde (MDA) and lead (Pb) in the blood serum were significantly greater, but activities of superoxide dismutase (SOD), glutathione (GSH) and concentrations of calcium (Ca) and magnesium (Mg) were lower in the serum of the exposed men. MDA, Pb, Ca and Mg were associated with the duration of exposure to handling e-waste. In males involved in handling of e-waste, there were 13 genes — ATM, ATR, ABL1, CHEK1, CHEK2, GADD45A, CDK7, GTSE1, OGG1, DDB1, PRKDC, XRCC1 and CCNH — for which expression of mRNA was up-regulated and 7 genes — BRCA1, GTF2H1, SEMA4A, MRE11A, MUTYH, PNKP and RAD50 — for which the expression of mRNA was down-regulated.
Conclusions: A strong correlation between indicators of damage of DNA, which could result in instability of the

Abbreviations: CA, chromosome aberrations; CBMN, cytokinesis-block micronucleus; CRTs, cathode-ray tubes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC–MS, gas chromatographic–mass spectrometry; GO, gene ontology; GSH, glutathione; HE, hematoxylin-eosin; MDA, malondialdehyde; OTM, olive tail moment; PBDEs, polybrominated diphenyl ethers; PCB, polychlorinated biphenyls; POPs, persistent organic pollutants; SOD, superoxide dismutase; SPE, Solid-phase extraction; TDNA%, the percentage of DNA in the comet tail; TM, tail moment

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genome, and duration of processing e-waste was observed. If proper procedures are not followed, there are significant risks to the health of the individuals involved in such activities.

1. Introduction

The global use of electronic equipment has increased dramatically over the last few decades, especially during the first decade of the 21st century. Electronics such as cell phones and computers are now used in all facets of life and provide significant benefits to society. According to Moore's Law, the microprocessor transistor count has doubled every one to two years since the early 1970s. This has resulted in electronics, especially personal devices, becoming obsolete at an exceedingly rapid pace. As a result, the production of e-waste has been increasing at approximately 4% per year (MH Wong et al. 2007) and has become the fastest growing waste stream in the industrialized world with approximately 20 to 50 million tons of e-waste being generated worldwide every year (Kumar and Singh, 2014; Lorenzen, 2014; Pramila et al., 2012). Some of the constituents in electronics are rare or valuable and thus are extracted for re-use. However, managing and recycling e-waste has become a major global challenge and environmental problem (Liu et al., 2009a; Tetteh and Lengel, 2017). In more economically developed countries, concerns about the potential effects of e-waste on human health and the environment have resulted in the export of e-waste to less developed countries, which may have less restrictive regulations. In fact, approximately 50 to 80% of e-waste produced globally is exported to Asia and Africa (Breivik et al., 2014; Daum et al., 2017; Ruan and Xu, 2016). Thus, the handling of e-waste is a global issue and has become one of social justice as well as being an economic issue.

Recently, an area near Tianjin, Northern China has become one of the largest centers for the recycling of electronic waste in China (Liu et al., 2009a). Due to a lack of policies to manage and control the recycling of e-waste in developing countries, much of this waste is processed in small-scale, unregulated family workshops where laborers manually disassemble electronics (Li et al., 2008; Liu et al., 2009b). The components of this waste, including electronic circuit boards or microchips, are then illegally burned so that valuable constituents can be extracted and reclaimed (see Fig. 1 in Ref [Du et al., 2018]). These procedures result in the release of a variety of chemicals to the environment. These contaminants are released primarily directly to the air but are also released directly and indirectly to the soil and water (Chatterjee, 2008; Leung et al., 2011). Among others, contaminants of concern released during the recycling of e-waste are metals such as lead (Pb), persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), and dioxin-like compounds, resulting in a variety of health risks (Chatterjee, 2007; Dallaire et al., 2014; Leung et al., 2011; CS Wong et al. 2007; Wu et al., 2009; Zhang et al., 2010). During these processes, organic and inorganic flame retardants, including those that are brominated such as polybrominated diphenyl ethers (PBDEs) or newer replacements are also released. For example, concentrations of Pb in the blood of children in e-waste recycling areas in China were significantly greater than those in the blood of children from non-e-waste recycling areas (Zheng et al., 2008). In fact, the exposure is approaching levels known to adversely affect the cognition and synthesis of heme in blood cells (Chen et al., 2011; Huo et al., 2007; Lu et al., 2017; Reglero et al., 2009b). Furthermore, a recent study by our groups has shown that local residents in the e-waste recycling industry of the Tianjin region exhibited oxidative stress-related health effects due to exposure to pollutants released during recycling (Li et al., 2013).

Despite concerns regarding potentially adverse effects on health due to participation in family-operated workshops, where the recycling of e-waste is often conducted in China and other countries in Asia and Africa, little is known about the specific effects on humans and their association with contaminants released during these practices. Most of the pollutants can damage DNA, which then can result in instability of the genome of workers or residents in the area where e-waste is recycled. The mechanisms of all of these effects are still unclear, but genetic toxicity from pollutants released during the processing of e-waste might be due to oxidative damage to nucleobases, induction of membrane lipid peroxidation, DNA methylation, and dysfunction of DNA repair, all of which can lead to human genetic instability (Singh et al., 2007). In an earlier study, it was demonstrated that people living in the e-waste recycling region of Tianjin exhibited cytogenetic damage of lymphocytes (Liu et al., 2009a). The aim of this study was to expand on previous investigations by conducting a comprehensive analysis of the potential health risks to humans working in e-waste disposal and recycling. Specifically, the quantity and quality of semen, profiles of gene expression, and cytogenetic effects, including chromosome aberrations, micronuclei in lymphocytes and DNA damage in both lymphocytes and spermatocytes were investigated.

2. Methods

2.1. Study population and sampling

The “exposed” group consisted of 146 male residents who were directly engaged in recycling e-waste but had no history of occupational exposure to other chemicals. All subjects from the exposed group were involved in dismantling e-waste on a daily basis, including melting and burning, during which vapors may have been inhaled. They all processed e-waste in similar family-workshops. The e-waste that they recycled mainly included discarded computers, TV sets and cathode-ray tube (CRTs) monitors. The reference group consisted of 121 men from a neighboring area located fifty kilometers from the exposure area, in which there was no recycling of e-waste or other chemical, industrial and agricultural pollution in the proximate vicinity (Liu et al., 2009a). The people in the reference group were all farmers who had planted vegetables, fruits and crops for many years. The demographic characteristics were determined via questionnaires administered to the subjects in the two groups (Table 1). In a previous study, it had been demonstrated that no significant cytogenetic damage occurred in subjects from this reference area (chromosome aberration: 1.70%) (Liu et al., 2009a) compared to the normal range (chromosome aberration: 1.61–3.30%) for Chinese people (Bai et al., 1993).

Peripheral blood and semen were collected from the two groups. To reduce variability among the results, only semen from men who had abstained from sexual activity between 2 and 7 days prior to collection were included in this study. All participants were instructed concerning the collection of semen by masturbation in a private and relaxed setting at Tianjin Medical University. Within 40 min of collection, analyses of the sperm were conducted in the genetics laboratory of Tianjin Medical University. In addition, a sample (10 ml) of venous blood was collected from each man, and concentrations of PCBs; trace elements, including Pb, copper (Cu), zinc (Zn), Ca, Mg, iron (Fe) and selenium (Se); chromosome aberrations (CA); cytokinesis-block micronucleus (CBMN); gene expression profiles; and DNA damage were measured. The Peking Union Medical College's Institutional Ethics Board approved all study protocols. Prior to the study, written informed consent was obtained from all subjects. All samples of blood from each group were used for the study of biomarkers. Samples of semen were used in routine semen analyses and in the comet assay.

2.2. Quantification of trace elements

Concentrations of Pb in the serum were determined by atomic absorption spectroscopy, as described previously (Reglero et al., 2009b). In brief, 100 µl of serum were added to 700 µl of 1 mmol/l HNO_3, followed by an addition of 50 µl of 0.02 mol/l HNO_3. Pb was then quantified with a graphite furnace-atomic absorption spectroscopy system (Shimadzu AA-660 AAS, GFA-4B graphite furnace atomizer and an ACS-60G autosampler Shimadzu Corporation, Kyoto, Japan). Concentrations of Cu Zn, Ca, Mg, Fe and Se in the serum were determined with flame atomic absorption (Shimadzu AA-660 AAS and an ACS-60G autosampler Shimadzu Corporation, Kyoto, Japan). The precision of the method was assessed by repeated analyses of standard solutions.

2.3. Quantification of PCBs

Concentrations of PCB congeners (Ballschmitter and Zell nomenclature) 8, 18, 28, 44,52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 170, 180, 187, 189, 195, 206 and 209 were quantified and analyzed with gas chromatography mass spectrometry (GC–MS). The extraction and cleanup of PCBs from whole blood was done using Solid-Phase Extraction (SPE). A 0.4 ml sample of blood was drawn into a vacutainer and placed into a centrifuge tube and mixed with 5% Na_2SO_4 (1 ml), acetonitrile (1 ml) and 85% isopropanol (1 ml), followed by whirl mixing for 1 min and ultrasonic treatment for 20 min. The tube was centrifuged at 16770*g* for 10 min. The supernatant was transferred to an activated SPE cartridge (C18, 60 mg/3 ml, Dikma Technologies, USA) and eluted slowly through the column with vacuum. Washing with 10 × 1.0 ml water was followed by elution with 3 × 1 ml n-hexane and 1 ml of a mixture of 20% dichloromethane and 80% n-hexane. The eluent was collected and evaporated at 40 °C under a gentle flow of nitrogen. The residue was redissolved in 200 µl n-hexane followed by addition of 200 µl concentrated sulfuric acid and whirl mixing for 1 min. The solution was allowed to stand for a few minutes until several layers formed. An aliquot of 100 µl in the n-hexane layer was injected into the GC–MS system (GCMS-TQ8040, Shimadzu, Japan). Separation was performed on a dual column system with an HP5-MS (30 m, 0.25 mm ID, 0.25 µm film thickness, Agilent Technologies, Palo Alto, CA, USA). Quantities of PCBs were determined with an external standard curve. The standard containing 28 PCB congeners was purchased from AccuStandard, USA.

2.4. Gene expression analysis

The mRNA of peripheral blood cells was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. The integrity of the RNA was assessed with the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Eighty-four key genes (see Table 1 in Ref [Du et al., 2018]) from the Human DNA Damage Signaling Pathway were simultaneously assayed with the RT^2Profiler PCR array plate (SuperArray Bioscience Corporation, Frederick, MD) according to the manufacturer's protocol. Briefly, samples were processed according to the manufacturer's instructions as Kasper described (Kasper et al., 2007). Signals were detected through chemiluminescence assay. For analyzing gene spot intensities of scanned X-ray films, the program ScanAlyze (Eisen Lab, Berkeley, CA) was employed.

Here, the GEArray Analyzer software (SuperArray) was used for background correction. Each array experiment was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as an internal standard). A detection limit of 0.05 times the GAPDH signal was determined. Data from four donors were determined in independent experiments.

Significantly changed genes were subsequently analyzed using IPA 5.0 (Ingenuity Systems Inc., CA). The pathways analyses, which was predicted to be influenced by differentially expressed genes, were ranked in order of significance and were further analyzed by overlaying with biological processes and canonical pathways. An overlap p value < 0.05 indicates a statistically significant, non-random association between a set of genes in the dataset and a related function. The p-value of the overlap was calculated by the Fisher's Exact Test.

Heatmaps based on the microarray data were generated with Heatmap Illustrator (Wuhan,PR China), version 1.0 (Deng et al., 2014). Pearson's correlation coefficient was used to assess the dose dependence on the expression of mRNA for genes. Two main clusters showed genes that were up- or down-regulated.

Original data were log-transformed and analyzed for statistical significance via the nonparametric Kolmogorov-Smirnov test for statistical significance with a false discovery rate (FDR) correction (FDR < 0.10). The results of the output are displayed as volcano plots, where the statistical significance was determined with the Kolmogorov-Smirnov test (FDR < 0.10). Pink lines were used to indicate the area of no significant fold change (fold change < 2).

2.5. Assessment of DNA damage by comet assay

The comet assay, which is based on fragmentation of DNA during alkaline unwinding due to strand breaks and adducts, was used to detect DNA single strand breaks (SSB) in spermatozoa and lymphocytes as previously reported and detailed in the supporting information (Haines et al., 1998). Spermatozoa and lymphocytes were suspended in PBS at a concentration of 1×10^5/ml, and damage to DNA by comet assay was performed as described previously (Liu et al., 2009a). All comet images were analyzed with the Comet Assay Software Project (CASP, Wroclaw University, Poland) (Końca et al., 2003), and the percentage of DNA in the comet tail (TDNA%), the tail moment (TM) and the Olive tail moment (OTM) were recorded to describe DNA damage to spermatozoa or lymphocytes. All experiments were performed in triplicate.

2.6. Chromosomal aberrations and analysis of micronuclei

Aberrations of chromosomes (CA) were quantified as described previously (Liu et al., 2009a). Briefly, peripheral blood was incubated in 4.5 ml RPMI 1640 medium containing 20% fetal bovine serum and PHA under 5% CO_2 at 37 °C for 48 h. Colchicine were added to make a final concentration of 0.06 µg/ml, and the cells were cultured for an additional 6 h. Lymphocytes were harvested for hypotonic treatment, fixed, and prepared as conventional slides for dyeing with Giemsa. Metaphase cells were scanned automatically with a ZEISS MetaSystem (Germany). For each subject, 100 metaphase cells were scored by two trained technicians. All aberrations, including acentric fragments, minute chromosomes, acentric rings, dicentric chromosomes, ring chromosomes, monomers, satellites, triradials and quadriradials were recorded, summed and reported as total aberrations.

To detect micronuclei specifically and efficiently, the cytokinesis-block micronucleus (CBMN) assay was used as described previously (Liu et al., 2009a). Binucleated lymphocytes were scanned automatically using a ZEISS MetaSystem for each slide. Micronuclei in 1000 binucleated lymphocytes per subject were scored.

2.7. Routine semen analyses

The semen was analyzed in accordance with standard procedures as described in the World Health Organization “WHO laboratory manual for the examination and processing of human semen” (World Health Organization, 2010). Assessment of motility was performed at 37 °C using a warmed microscope stage. At least five microscopic fields at a magnification of 40× were assessed to classify 200 spermatozoa. The motility of each sperm was graded as “a”, “b”, “c” or “d” according to the classification standard provided in the manual (Haines et al., 1998): a = rapid progressive motility; b = slow or sluggish progressive motility; c = non-progressive motility; and d = no motility. The percentage of grades a + b was recorded for the statistical analysis. The proportion

of motile sperm was calculated as (a + b)/(a + b + c + d) × 100%.

The defects of spermatozoa were assessed by making a seminal smear on a glass slide with 5 μl of semen. The sperm morphology was then assessed after staining air-dried smears of spermatozoa with hematoxylin-eosin (HE) (Sigma-Aldrich, Inc., St. Louis, USA). Defects of the head, neck, mid-piece and tail, or as indicated by the presence of cytoplasmic droplets, were noted. The sperm morphology was assessed by a technician using the strict morphology method recommended by the WHO (World Health Organization, 2001) and in which only sperm with no defects are classified as normal (Guzick et al., 2001; World Health Organization, 2010; Swan et al., 2003). The normal reference values for semen parameters were based upon those reported previously (Gao et al., 2006; Gao et al., 2008; Swan et al., 2003).

2.8. Indicators of oxidative stress

Levels of oxidative stress biomarkers in serum, including malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD), were quantified as described by Reglero et al. (Reglero et al., 2009b) using commercial kits (JianCheng, Inc. Nanjing, China) with an automated spectrophotometer (ND8000, NanoDrop, USA).

2.9. Statistical analyses

The data were analyzed using the Graphpad Prism 6.01 software and SPSS software package 17.0 (SPSS, Chicago, IL, USA). Normality was confirmed with the Kolmogorov-Smirnov test, and the homogeneity of variance was confirmed with Levine's test. Concentration of PCBs was logarithmically transformed to obtain more normal distributions, and log-transformed normal distributions were then confirmed with the Kolmogorov–Smirnov test. The differences between groups were examined with the independent samples *t*-test. The Crosstabs Chi-square test was used to analyze the demographic characteristics, CA, and CBMN of the two groups. The t-test was used to compare the TDNA%, TM, OTM, oxidative stress biomarkers and elements (except Pb) between the exposure and reference groups. Concentrations of Pb and several semen parameters did not meet the assumption of homogeneity of variance, so the data were analyzed with a non-parametric Wilcoxon rank-sum test. A two-way ANOVA was used to test for interactions between smoking habits and semen quality, respectively. A multivariate logistic regression analysis of risk factors for sperm motility rate, abnormality rate and total sperm count was performed. Regression coefficients were calculated by the adjustment of covariates: age, smoking, education, and family income. A P value < 0.05 was considered statistically significant.

3. The results

3.1. Demographic characteristics

A crosstabs analysis revealed that there were no significant differences in the demographic characteristics between the two experimental groups (Table 1). Specifically, no statistically significant difference occurred for age, nationality, smoking status and days of sexual abstinence. However, the family incomes of subjects from the exposed group were significantly greater because they made additional money from e-waste recycling jobs.

3.2. Trace elements in serum

Concentrations of Pb were significantly greater, but those of Ca and Mg were significantly lower in individuals who worked with e-waste compared to concentrations in the serum of individuals in the reference group (Fig. 1A, B). There were no statistically significant differences in concentrations of Cu, Zn, Fe or Se in the serum between the two groups. Because concentrations of Pb, Ca and Mg in the serum of e-waste workers were different from those of the reference subjects, relationships among Pb, Ca and Mg and duration working with e-waste were analyzed. Concentrations of Pb increased proportionately with the duration working with e-waste, and concentrations of Ca and Mg were inversely proportional to the duration working with e-waste (Fig. 1C).

Table 1
Demographic characteristics of study participants by group [*n* (%)].

Covariate	Exposed group (n = 146)	Reference group (n = 121)	P value (χ^2)
Exposure duration (years)			
≤ 3	63(43)	0	
3–6	53(36)	0	
> 6	30(21)	0	
Age (years)			0.906(0.198)
20–29	41(28)	36(30)	
30–39	80(55)	63(52)	
> 40	25(17)	22(18)	
Average	35.8	34.9	
Nationality			0.157(2.005)
Han	139(95)	119(98)	
Non-Han	7(5)	2(2)	
Education			0858(0.306)
Middle school or less	99(68)	80(66)	
High school	45(31)	40(33)	
College	2(1)	1(1)	
Family income (US$/year) conversion from RMB			0.000(32.212)
≤ 5000	13(9)	17(14)	
5001–10,000	39(27)	68(56)	
≥ 10,001	94(64)	36(30)	
Smoking status			0.764(0.090)
Yes	105(72)	85(70)	
No	41(28)	36(30)	
Days of sexual abstinence			0.515(0.424)
2–3	89(61)	69(57)	
4–7	57(39)	52(43)	

3.3. PCB congeners in whole blood

The mean total concentrations of 28 congeners of PCBs in the blood of exposed individuals was significantly ($P < 0.001$) greater than that of the control. Concentrations of individual PCB congeners in the group of workers who recycled e-waste were significantly ($P < 0.001$) greater than those of the respective congeners in the blood of the reference group (Fig. 2A). Constituent PCB congeners varied more in the group of people working in recycling e-waste than in the reference group (Fig. 2B, C).

3.4. DNA damage in lymphocytes and spermatozoa

The damage to DNA represented by TDNA%, TM and OTM, as determined in the comet assay, was significantly greater for subjects involved with e-waste recycling activities than those of the reference group for both lymphocytes and spermatozoa (Fig. 3). Since age might affect the condition of the DNA in the lymphocytes and spermatozoa, the exposure and reference group were both stratified by age into three sub-groups (20–29, 30–39 and > 40 years old). For each sub-group based on age, significant differences in the damage of DNA in lymphocytes and spermatozoa were observed between persons involved in recycling e-waste and those who were not (see Fig. 2 in Ref [Du et al., 2018]). No significant difference in damage to DNA was observed among age groups in either the exposure or reference group. The results of the two-way ANOVA also showed that the factor “age” had no significant ($P > 0.05$) interactions with exposure to contaminants during the recycling of e-waste.

To analyze the relationship between damage to DNA and duration of exposure, the group of workers who recycled e-waste was stratified into three sub-groups based upon the duration they worked in e-waste

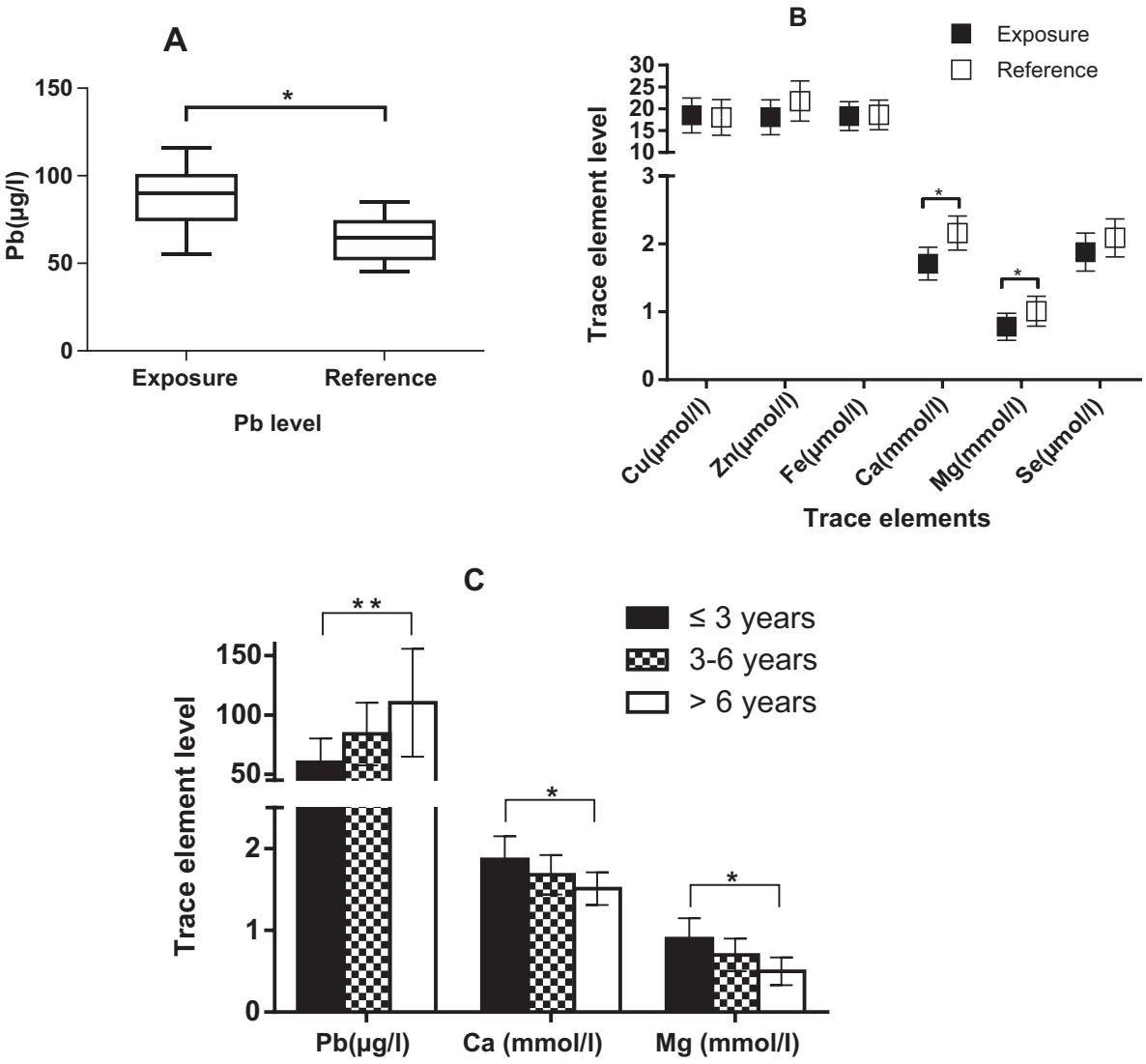

Fig. 1. Trace elements of exposed and reference groups (A,B) and relationship between the level of Pb, Ca, Mg and exposure duration (C). The concentration of Pb in the blood of workers who recycled e-waste was greater than that of the reference group (Wilcoxon rank-sum test). B: Concentrations of Cu, Zn and Fe, Ca, Mg and Se in the blood of workers who recycled e-waste and reference groups. Concentrations of Ca and Mg were lower in the blood of workers who recycled e-waste than that of individuals in the reference. No difference was found for the other elements between the two groups. C: Relationship among Pb, Ca, Mg level and exposure time. Concentrations of Pb increased, but concentrations of Ca and Mg decreased as a function of duration working with e-waste. **: $P < 0.01$, *: $P < 0.05$.

recycling (≤3, 3–6 and > 6-year groups). This analysis revealed statistically significant relationships between DNA damage (TDNA%, TM) and duration of exposure for DNA damage in both the lymphocytes and spermatozoa (see Fig. 3 in Ref [Du et al., 2018]).

Smoking might also damage the DNA of lymphocytes or spermatozoa. Thus, the exposure and reference groups were stratified into two sub-groups, classified as smokers or non-smokers. In the reference group, subjects that smoked exhibited more damage to the DNA of lymphocytes than individuals who did not smoke. No differences in the amounts of damage to the DNA of lymphocytes or spermatozoa were observed between smoking and nonsmoking subjects in the exposed group (Fig. 4). The results of the two-way ANOVA also showed that smoking habits had no effect on exposure to e-waste ($P > 0.05$).

3.5. CA and CBMN

Aberrations in DNA, as expressed by total CA or CBMN, were significantly ($P = 0.000$) greater in persons working in e-waste recycling than those in the reference group (Table 2). CA and CBMN were also analyzed in three sub-groups stratified by age in both the e-waste handlers and reference groups. For each of the sub-groups stratified by age, there was significantly greater damage to the DNA of the e-waste recyclers compared to the reference group (see Fig. 4 in Ref [Du et al., 2018]). No significant difference was found among sub-groups in either the exposure or reference group. The results of the two-way ANOVA also showed that age had no significant ($P > 0.05$) association with exposure during the recycling of e-waste. The relationships between CA, CBMN and the duration of working with e-waste were also investigated. The group working in the recycling of e-waste was divided into three sub-groups based on duration of exposure (≤3, 3–6 and > 6 years). Statistically significant, positive associations were observed between both CA and CBMN and the duration of working with e-waste (see Fig. 5 in Ref [Du et al., 2018]).

Smoking might be associated with chromosomal damage in lymphocytes; thus, the exposure and reference groups were both stratified into two sub-groups of either smokers or non-smokers. In the reference group, smoking subjects exhibited more CBMN in lymphocytes than non-smokers. However, no difference was observed for CA or CBMN

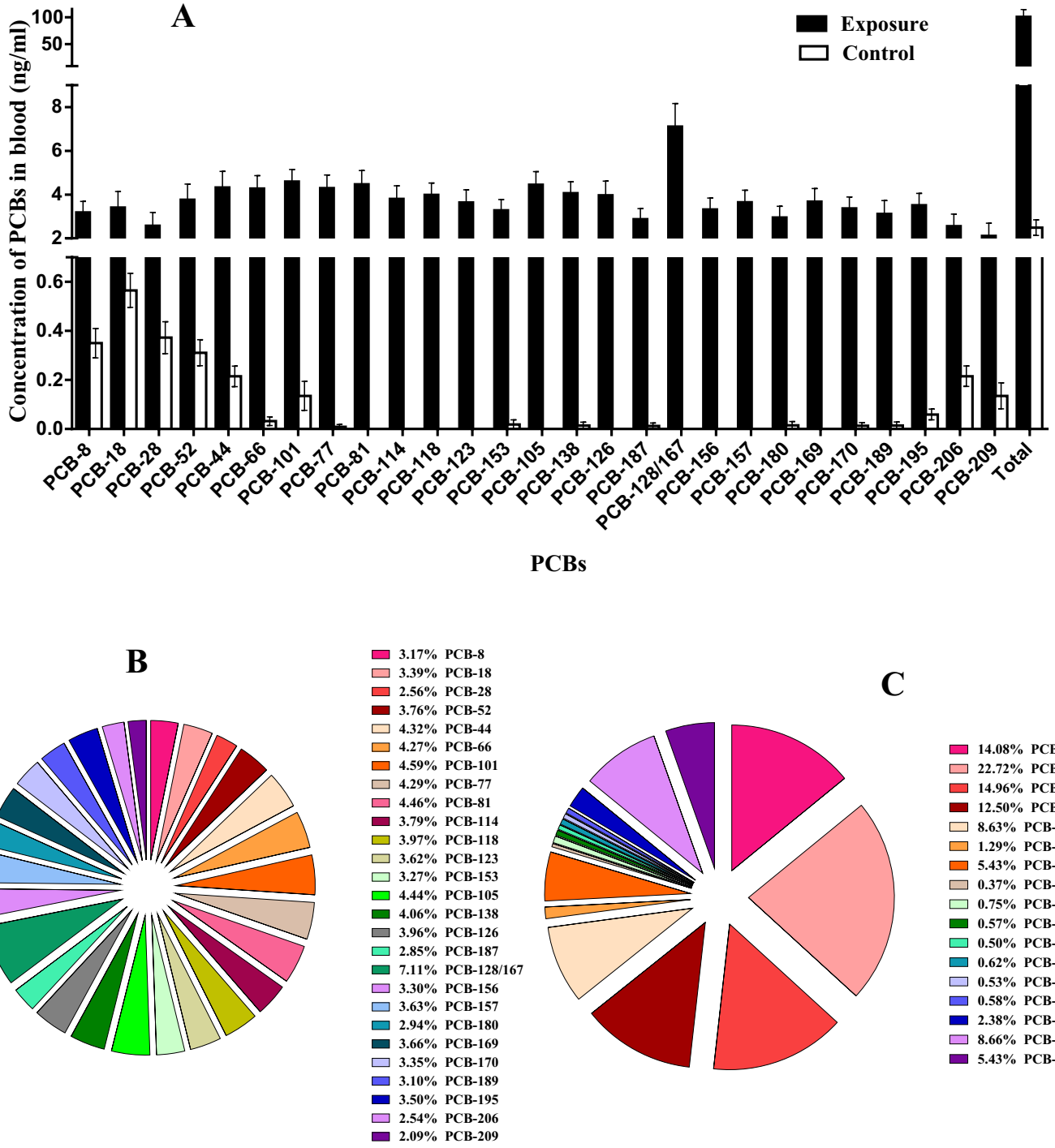

Fig. 2. Exposure of major PCB congeners in human blood samples from exposure and reference areas. A: Concentration of PCB congeners blood of the group of workers who recycled e-waste and reference groups. B and C: Constituent and proportion of PCB congeners in the group of workers who recycled e-waste and reference groups.

between smoking and nonsmoking subjects in the exposed group (Fig. 5). The results of the two-way ANOVA ($P > 0.05$) also showed that smoking habits had no interaction with the exposure of e-waste.

3.6. Semen analysis

Statistically significant differences in the quality of sperm were observed between the group handling e-waste and the reference group. The mean volume of semen and the total number of spermatozoa in individuals handling e-waste were significantly less than those in individuals from the reference group. However, there was no statistically significant difference in the sperm concentration or pH between the two groups (Table 3). Motility and morphological abnormalities of sperm were statistically significantly less and greater, respectively, in semen collected from men handling e-waste compared to the reference group. The results of the two-way ANOVA analysis revealed that smoking habits were not significantly associated with exposure to e-waste and did not confound the effects of exposure to e-waste ($P > 0.05$) (Table 3).

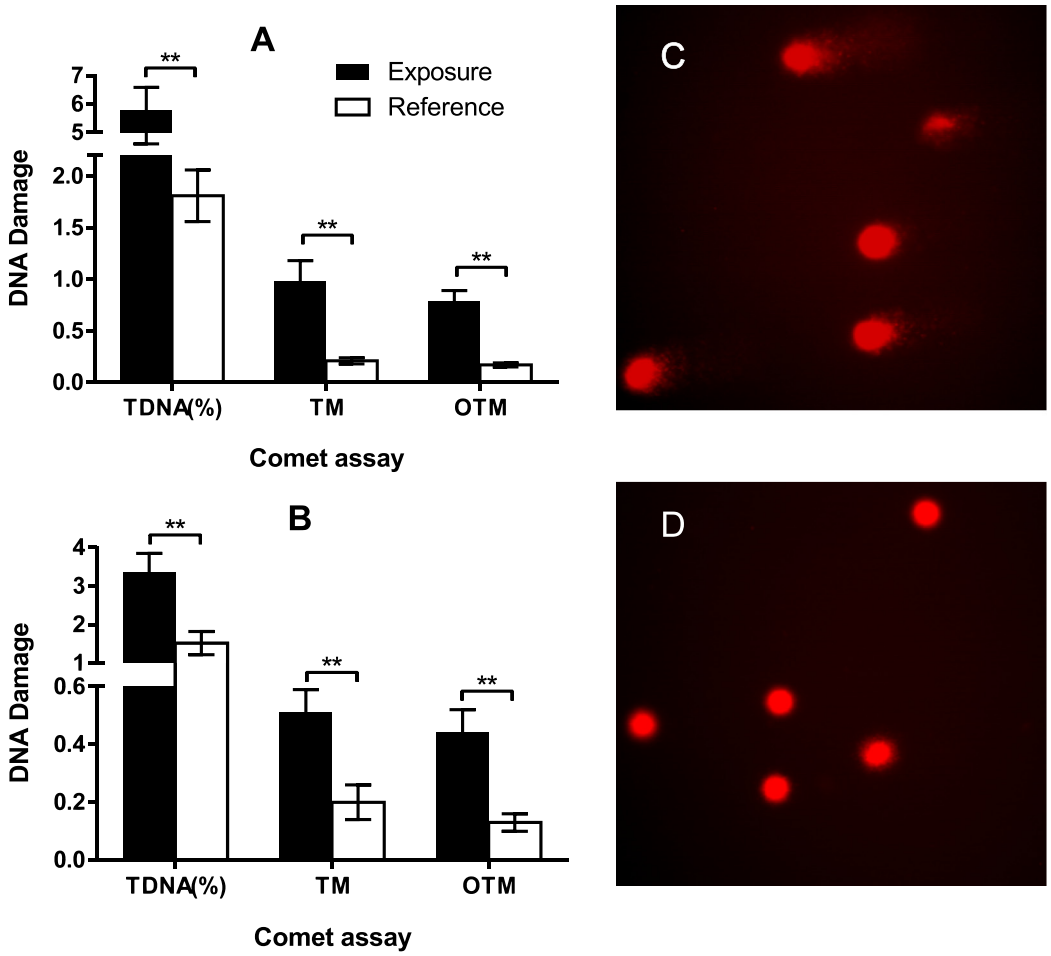

Fig. 3. DNA damage of lymphocytes (A) and spermatozoa (B) in men in the exposure and reference groups detected by the comet assay. A and B: TDNA%, TM and OTM of the lymphocytes and spermatozoa in the group of workers who recycled e-waste are all much higher than those of the reference group. C: Comet image of lymphocytes from the exposed group. The comet tail shows DNA damage in the lymphocytes. D: Normal lymphocytes from the reference group. **: $P < 0.01$.

The effects on the motility of sperm, morphological abnormalities and total number of spermatozoa were analyzed in the three sub-groups stratified by age to compare between the group handling e-waste and the reference group. When stratified for age, for each of the age sub-groups, a statistically significant difference was observed between the group exposed to e-waste and the reference group (see Fig. 6 in Ref [Du et al., 2018]). Motility, morphological abnormalities and the total number of spermatozoa were also different among sub-groups in the exposure or reference group, respectively.

Relationships between measures of semen quality and duration of working with e-waste were investigated by stratifying the three sub-groups by duration of exposure (≤3, 3–6 and > 6 year groups), and the semen parameters were compared between individuals who worked with e-waste and the reference group. The volume of semen and number and motility of spermatozoa were inversely proportional to the duration of handling e-waste, but incidences of abnormalities were positively correlated with the duration of exposure during the recycling of e-waste (see Fig. 7 in Ref [Du et al., 2018]).

3.7. Biomarkers of oxidative stress

Concentrations of MDA, GSH and SOD in the serum were significantly different between individuals who handled e-waste and those who did not (Fig. 6A). Concentrations of MDA were significantly greater in men from the group of e-waste recyclers than those in individuals of the reference group. In contrast, the activities of GSH and SOD were significantly lower in individuals recycling e-waste compared to those in individuals from the reference group. When individuals handling e-waste were stratified into three sub-groups based upon the exposure time (≤3, 3–6 and > 6 year groups), there was a statistically significant relationship between concentrations of MDA in the serum and duration of working with e-waste (Fig. 6B). However, there were no statistically significant relationships between activities of GSH or SOD and duration working with e-waste.

3.8. Gene expression profiling in peripheral blood lymphocytes

In a previous study (Liu et al., 2009a) and the present study, it was found that there was significant DNA damage in workers exposed to e-waste (Figs. 3 and 4). Therefore, it was hypothesized that some key genes related to the damage and/or repair of DNA might be altered in individuals in the group exposed to e-waste. To test this hypothesis, the RT^{2}Profiler PCR array was used to measure levels of expression of those genes related to quality of DNA. Men in the e-waste recycling group displayed 13 up-regulated genes (solid line box), including ATM, ATR, ABL1, CHEK1, CHEK2, GADD45A, CDK7, GTSE1, OGG1, DDB1, PRKDC, XRCC1, and CCNH, and 7 down-regulated genes (dashed line box), including BRCA1, GTF2H1, SEMA4A, MRE11A, MUTYH, PNKP, and RAD50, compared to the reference group (Fig. 7B). The expressions of up-regulated genes exhibited dose-effect relationships associated

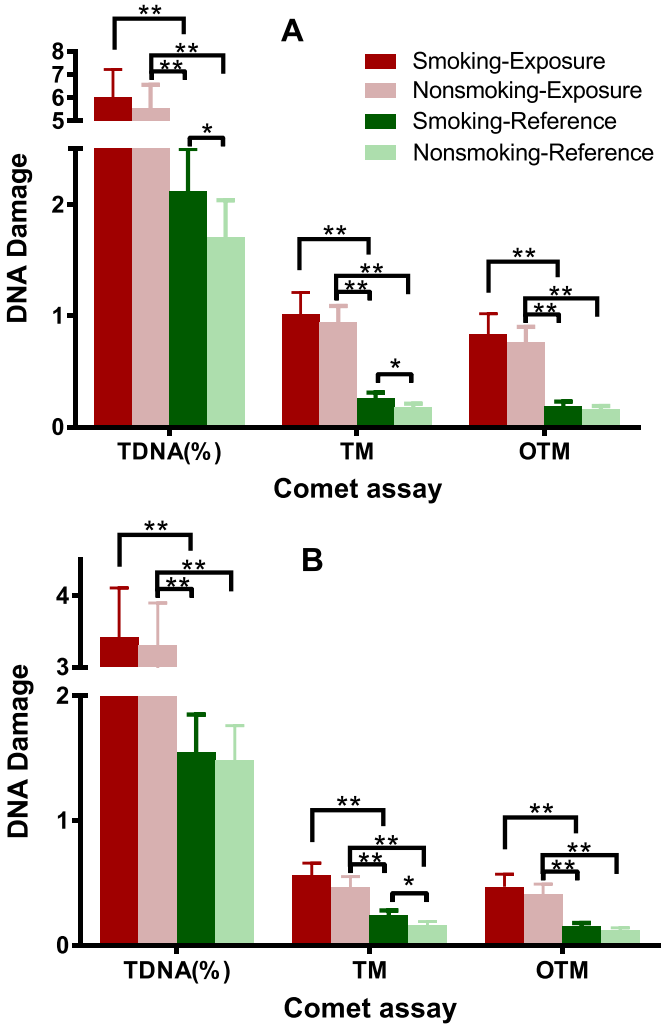

Fig. 4. DNA damage measured by the comet assay in lymphocytes (A) and spermatozoa (B) from men as smoking and nonsmoking subjects. TDNA%, TM in lymphocytes (A) and TM in spermatozoa (B) of smoking subjects were greater than those of nonsmoking subjects in the reference group. However, there was no difference of DNA damage between smoking and nonsmoking subjects within the group of workers who recycled e-waste, not only in lymphocytes but also in spermatozoa. TDNA%, TM and OTM of both smokers and nonsmokers in the group of workers who recycled e-waste were greater than these same parameters in the reference group. **: $P < 0.01$, *: $P < 0.05$. A two-way ANOVA was also used to test the interactions between smoking and DNA damage in the lymphocytes and spermatozoa, respectively. (Lymphocytes: $F = 1.25$, $P = 0.33$; spermatozoa: $F = 1.49$, $P = 0.29$.)

with e-waste exposure duration. The GO analysis showed that the genes were predominantly involved in the biological processes and pathways, including genome stability, cell cycle, apoptosis, stress response, p53 pathway and repair of DNA (Fig. 7A). Genes clustered in the heat map with > 2-fold up-regulation and > 2-fold down-regulation were shown in Fig. 7C.

3.9. Assessment of risk factors

Significant and positive correlations were found between SOD or GSH and the quality of semen (Table 4). Significant negative correlations were observed between the quality of semen and total concentrations of PCBs and MDA. Total concentrations of PCBs, MDA and Pb were positive, but GSH and SOD were negatively associated with duration of exposure (Table 4). MDA was positively associated but SOD and GSH were negatively associated with concentrations of Pb (Table 4). Negative correlations were also found between Pb and Ca ($R = -0.615$, $P = 0.000$) and Pb and Mg ($R = -0.458$, $P = 0.013$) (data not shown). A multivariate logistic regression analysis of risk factors revealed that exposure time, total PCBs, MDA and Pb were predominant risk factors for the quality of semen (Table 5).

Table 2
CA and CBMN of lymphocytes in the exposure and reference groups.

Group	Subjects	Cells	Total aberrations/ micronucleus	CA rate/ CBMN rate	χ^2	p
CA						
Exposure	146	12,591	1008	8.01%	444.5	0.000
Reference	121	10,361	186	1.80%		
CBMN						
Exposure	146	123,309	3243	26.30‰	1614.0	0.000
Reference	121	100,358	454	4.52‰		

4. Discussion

The results of this study demonstrated the potential effects on male reproductive health associated with occupational exposure of men from the Tianjin region involved in the recycling of e-waste. A range of inorganic and organic chemicals can be released into the ambient environment during the recycling of e-waste (Song and Li, 2015), especially with primitive techniques in small, informal workshops that do not follow prescribed procedures using the latest safety and personal protection equipment. Workers at unlicensed, unsupervised e-waste recycling facilities can be exposed to contaminants, as was demonstrated by the significantly elevated concentrations of Pb and PCBs in blood plasma observed during this study (Heacock et al., 2016; Yang et al., 2013; Zhao et al., 2010) and can suffer adverse effects on health (He et al., 2017; Song and Li, 2014). To the best of our knowledge, the study — the results of which are presented here — was the first to assess associations between recycling e-waste and the quality and quantity of semen as well as cytogenic damage in peripheral blood and the spermatozoa of adults exposed to pollutants during the recycling of e-waste.

Damage to DNA in lymphocytes and spermatozoa observed in this study, with the comet assay and greater frequencies of CA and CBMN in individuals exposed to e-waste, are consistent with findings from a previous study investigating human lymphocytes that reported significantly greater damage to DNA and aberrations of chromosomes in lymphocytes of people living in regions where the recycling of e-waste was being conducted compared to persons from a reference area (Liu et al., 2009a). Damage to DNA caused by exposure to contaminants released during the recycling of e-waste can be the result of a number of mechanisms of toxic action and pathways, including direct oxidative damage to DNA, induction of lipid peroxidation, DNA methylation, formation of adducts with DNA and dysfunction of repair of damaged DNA, all of which can lead to genomic instability in humans (Singh et al., 2007; Song and Li, 2014). A significant induction of markers of oxidative stress, such as concentrations of MDA — a product of peroxidation of lipids — in individuals working in the recycling of e-waste would support the hypothesis that oxidative stress was one of the main drivers of observed cytogenic damage in lymphocytes and sperm cells. Measures of antioxidant capacity, including activities of SOD and GSH, were lower than those in individuals from the reference group, which indicated a lessened capacity due to combating oxidative stressors, which resulted in chronic oxidant stress in vivo and an important hazard factor for DNA damage and cytogenetic alteration in e-waste recycling workers.

One of the main contaminants of concerns released during e-waste recycling processes is Pb — used in soldering to connect electronic

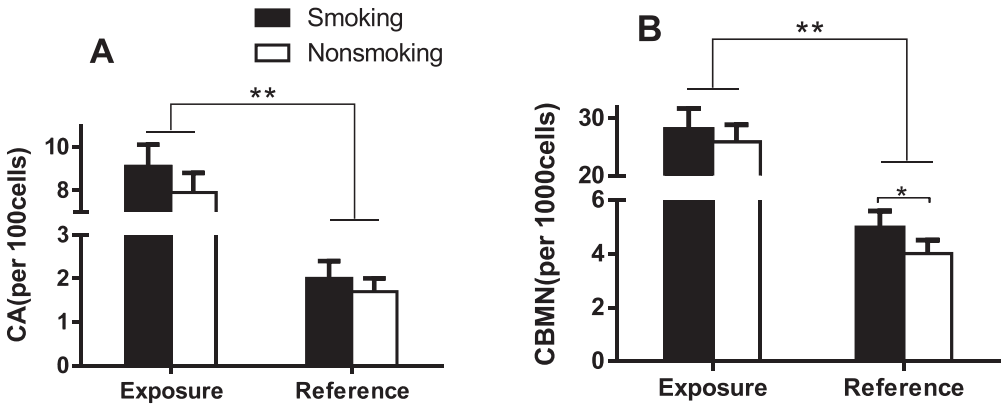

Fig. 5. CA (A) and CBMN (B) in the lymphocytes of smoking and nonsmoking subjects. The CBMN (B) of smoking subjects was greater than that of nonsmoking individuals in the reference group. However, there is no difference in CA and CBMN between smoking and nonsmoking subjects in the group of workers who recycled e-waste (A). The CA and CBMN rates of both smokers and nonsmokers in the group of workers who recycled e-waste were greater than those in the reference group. **: $P < 0.01$, *: $P < 0.05$. A two-way ANOVA was also used to test the interactions between smoking and CA and CBMN in lymphocytes, respectively. (CA: $F = 2.61$, $P = 0.11$; CBMN: $F = 3.05$, $P = 0.09$.)

Table 3
Two-way ANOVA analysis of semen quality between the two groups ($\bar{x} \pm$ SD).

Group	N	Semen Volume (ml)	pH	Sperm concentration (1×10^6/ml)	Motility rate (%)	Abnormality rate (%)	Total sperm count (1×10^6)
Exposure	146	1.39 ± 0.56**	7.28 ± 0.44	47.78 ± 18.29	45.01 ± 8.93**	31.11 ± 8.33**	102.50 ± 30.22*
Reference	121	2.54 ± 0.70	7.38 ± 0.08	44.77 ± 18.45	58.48 ± 9.32	21.77 ± 6.54	117.21 ± 32.03
P1		0.936	0.795	0.813	0.909	0.455	0.811

P1: *P* value of the interaction between smoking and exposure;
* $P < 0.05$, analyzed by the Wilcoxon rank-sum test for the two groups.
** $P < 0.01$, analyzed by the Wilcoxon rank-sum test for the two groups.

components to boards — which melts at temperatures used to reduce the volume of e-waste and release metals from plastics. It can be released as a vapor and is associated with repairable particles, which creates a particular health concern for e-waste recycling workers. In this study, concentrations of lead were significantly greater in the blood of individuals working in the recycling of e-waste, which confirmed exposure to this metal associated with the recycling of e-waste. In fact, they exceeded the upper reference range values of 50 μg/L set by the U.S. Centers for Disease Control set for children, which are based on earlier studies that have found neurobehavioral impairment in children with BLLs below 100 μg/L (Canfield et al., 2003; Lanphear et al., 1998). Furthermore, studies in humans that investigated the potential effects on male reproductive functions suggested that Pb exposures as low as 400 μg/L, which is only four times the average concentrations measured in e-waste recycling workers in this study, can decrease sperm counts and increase the frequency of abnormalities of spermatozoa (Alexander et al., 1996; Telisman et al., 2000). Furthermore, chronic exposure to Pb (independent of actual lead exposure levels) has also been associated with declining sperm concentrations and sperm motility (Alexander et al., 1996). In addition, significantly lower concentrations of Mg and Ca were observed in parallel with greater concentration of Pb in the serum of individuals working in the e-waste recycling industry compared to men in the reference group. Because Pb has a similar charge, the electronegativity, hydrated ionic radius and crystalline structure can isomorphically substitute for Ca and Mg to a lesser extent. Lead competes with the absorption and transport of Mg and Ca and causes an altered homeostasis of Mg and Ca (Cerklewski, 1983; Pounds, 1984; Simons, 1993). Similar to exposure to Pb, PCBs can affect the homeostasis of Ca. PCBs might promote the release of Ca^{2+} and block the Ca^{2+} influx from extracellular space[49]. These findings suggest that the homeostasis of Ca^{2+} was likely disrupted in men involved with e-waste recycling activities, which can result in adverse health consequences.

Pb is classified as a reproductive and/or neuroendocrine toxicant (Mendiola et al., 2011; Tapisso et al., 2009; Yan et al., 2013) that has been shown to cause oxidative stress in several tissues, including the blood, testes and spermatozoa (Marchlewicz et al., 2004; Reglero et al., 2009a; Tapisso et al., 2009). Oxidative stress affects male fertility because it damages the membranes of sperm cells, decreasing both the motility of sperm and its ability to fuse with the oocyte (Tremellen, 2008; Vernet et al., 2004). Thus, adverse health consequences associated with male-related effects on reproduction likely represent a critical effect in men involved with the recycling of e-waste.

In addition to Pb, concentrations of PCBs were significantly greater in the blood of workers who recycled e-waste compared to the reference group. PCBs were widely used as dielectric and coolant fluids in transformers, capacitors and electric motors. During the dismantling of e-waste, which included heating or burning, large quantities of PCBs are released into the environment where people work and live. Persistence and bioaccumulation potentials of congeners of PCBs enable them to be deposited in plants, fish and mammals, which can subsequently accumulate in human consumers (Grossman, 2013; Luo et al., 2007). Accumulated PCBs have been previously shown to cause toxicities to endocrine, immune, metabolic, reproductive, and nervous systems (Nishimura et al., 2017; Zheng et al., 2017). The adverse consequences of PCBs on reproduction can be synergistic with the effects of Pb.

While concentrations of PCBs were not considered to necessarily be the putative causative agents for adverse effects, they did represent a useful maker of overall exposure to contaminants from recycling e-waste. Any other possible contaminants were released during informal recycling e-waste processes in certain regions in China and are associated with adverse health effects in exposed people. For example, the greatest concentrations of dioxins and dibenzofurans (PCDD/Fs) reported for air have been measured in those areas (He et al., 2017; Li et al., 2007; Yu et al., 2006). Exposure to dioxins and dioxin-like chemicals has been previously associated with a series of health effects such as cancer, cognitive impairment, and developmental and reproductive dysfunction (He et al., 2017; Kogevinas, 2001; Peterson et al., 1993). Furthermore, exposure to plasticizers and BFRs during the

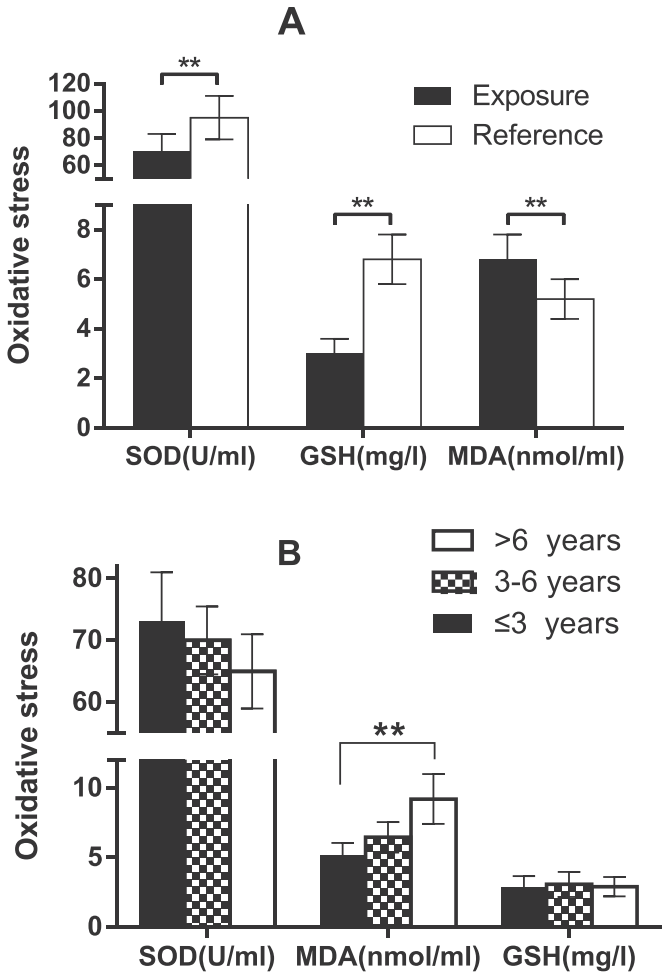

Fig. 6. The level of MDA, GSH, SOD in the serum between the two groups (A) and the relationship between MDA, GSH, SOD and exposure duration (B). A: SOD and GSH were significantly less, but MDA was significantly greater in the group of workers who recycled e-waste compared to those of the reference group. B: There was a significant, positive relationship between MDA and exposure duration. **: $P < 0.01$.

burning of e-waste is also of concern due to their endocrine-disrupting potential and the hypothesized adverse reproductive health effects of these chemicals (Covaci et al., 2011; Darnerud, 2008). Thus, the cytogenic and health effects observed during this work were likely due to the combinatory exposure to a complex mixture of contaminants released during e-waste recycling practices.

The observation that damage to DNA, as determined by significant increases in CA and CBMN, occurred as a function of duration of working in recycling e-waste is direct evidence for adverse effects on genetic materials due to working with e-waste. Clastogenic potentials were previously reported to be dependent on the duration of exposure (Tapisso et al., 2009), which is consistent with the effects of duration observed during this study. This time dependency is indicative of a potential contribution of bioaccumulative chemicals released during the recycling practices to the observed health effects. Based on the regression model, the duration of exposure was the main risk factor for the semen quality, and total PCBs, MDA and Pb were considered weaker but were also significant risk factors for the motility and abnormality rate of sperm. Contamination by processing e-waste had a greater contribution to male-related effects to lymphocytes and spermatozoa damage observed in the exposed group than smoking, which might be because the damage from exposure to handling e-waste was so significant that smoking did not significantly contribute to additional DNA damage.

Relatively large amounts of damage to DNA were exposed in the sperm of men who worked in recycling e-waste. They also exhibited impaired abilities to repair damaged DNA, which might be due to oxidative damage to nucleobases, the induction of membrane lipid peroxidation, and DNA methylation. Pollutants released during the processing of e-waste might disrupt the expression of some key genes that take part in DNA repair pathways in workers who recycled e-waste. All of the genes play a key role in maintaining stability in the genome via the cell cycle, apoptosis, stress response, p53 pathway and DNA repair. Therefore, those genes were apparently not efficient at repairing damaged DNA, as indicated by the greater rates of damage to DNA measured by the comet assay. These findings in the present study might be helpful for discovering the mechanisms of genome instability induced by e-waste exposure in the future.

There is an urgent need to better describe exposure to and the effects of mixtures of chemicals to which workers are exposed during the uncontrolled recycling of e-waste. It is recommended that the additional chemicals discussed above should be measured in human tissues, such as peripheral blood, cord blood, hair and urine, as well as environmental matrices, including soil, water and air (Grant et al., 2013). Furthermore, it is anticipated that contaminants released during the uncontrolled recycling of e-waste pose risks not only to the quantity and quality of semen in men but also to the health of women and children (Ben et al., 2013; Xu et al., 2012). These findings suggested that the outdated and unsafe methods that are currently used to recycle e-waste in many regions around the world pose significant hazards to human health and the general environment. The potential adverse health effects due to damage to DNA can be projected to other clinical outcomes that need to be investigated for the long term in the exposure scenario. Thus, when considering potential implications for people and the environment, further research concerning the applicability, effectiveness and efficiency of various processes and equipment for the management of e-waste is needed (Jung et al., 2015). Moreover, to reduce exposure to pollutants during the recycling of e-waste, the research and development of biodegradable and ecofriendly materials for electronics are also suggested. High-performance, green and biodegradable materials have recently been developed that might be useful in manufacturing electronics that are most likely to have relatively short service lives (Jung et al., 2015). Reasonable policies to improve the management of e-waste, especially in China and other developing countries, are needed (Brunner and Fellner, 2007; de Souza et al., 2016; Garlapati, 2016). However, changes to policies alone are not enough. The health education of workers involved in recycling e-waste should be performed as soon as possible because the health impact of multiple chemicals is likely to be significant. Reducing exposure is the ultimate goal for public health investigations. Furthermore, practical actions by all countries are the best way to solve the e-waste problem. In the future, we recommend a full examination of risks and enforcements of regulatory compliance of abandoned e-waste recycling areas. Since pollution from the recycling of e-waste has become a global environmental problem, a global solution to the effects of exposure to e-waste must become a priority in the international community.

5. Conclusions

A strong correlation between instability of the genome and duration of exposure to contaminants from handling and processing e-waste was observed. Thus, it can be concluded that, despite the benefits to society from recycling e-waste, if proper procedures are not followed, significant risks to human health will be present in such activities. As a consequence, it is necessary to implement additional measures to minimize the exposure of workers and local residents to contaminants released during the recycling of e-waste. Furthermore, workers should be monitored to ensure that they are not exposed to toxicants released during recycling activities.

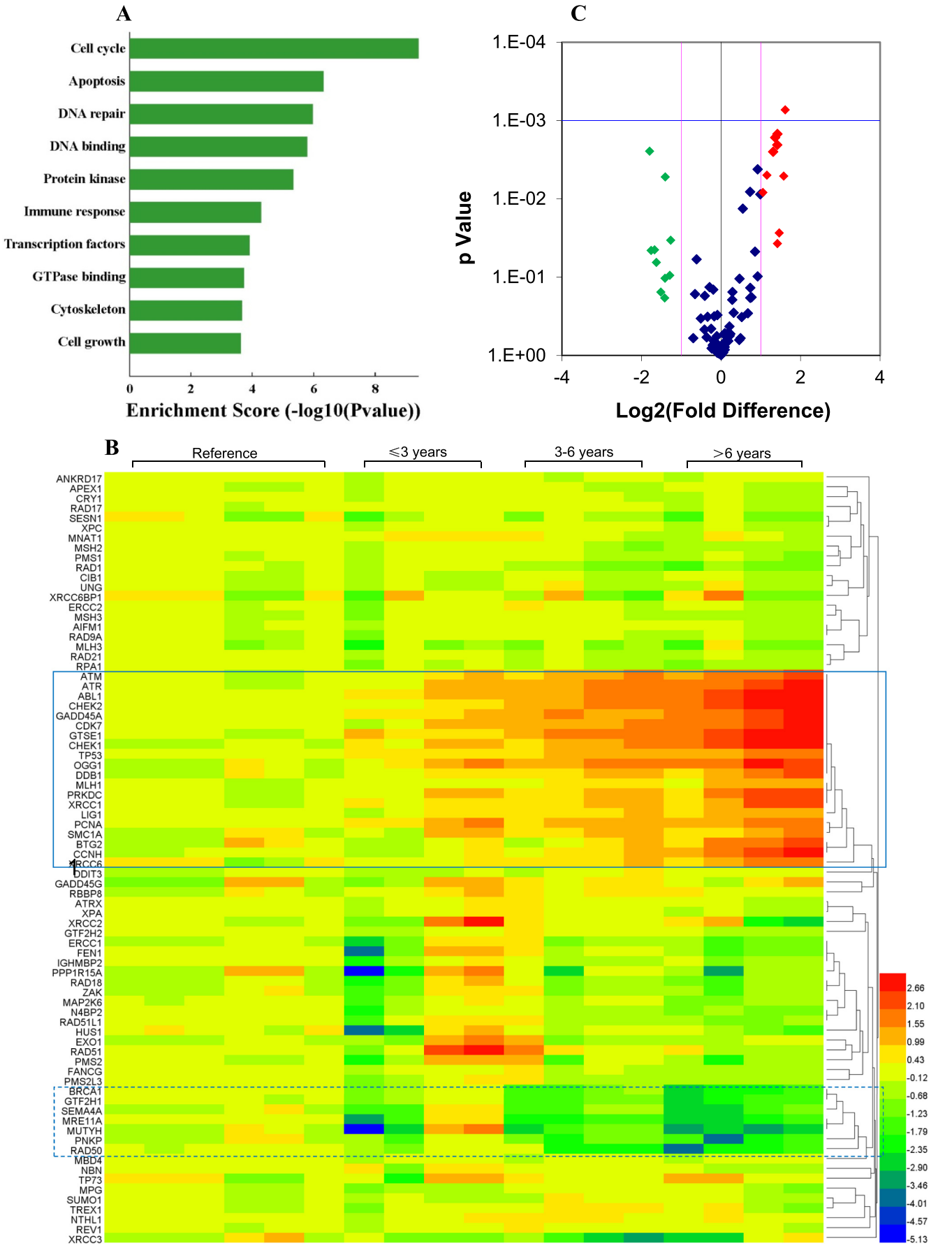

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Fig. 7. Identification of gene response to e-waste exposure by gene profile analysis. (A): A gene ontology annotation enrichment analysis was performed to elucidate the biological processes and pathways associated with each gene function in the group of workers who recycled e-waste. The top annotation clusters are shown in bar plots; the value of the abscissa reflects the annotation enrichment score. Group names on the ordinate were based on the biological processes and pathways. (B): A heat map illustrating a hierarchical cluster analysis was shown. Clusters were created using hierarchical clustering of the gene expression profiles, where each row represents an individual gene. Two main clusters showed genes that were activated (in the solid line box) and suppressed (in the dashed line box) in the group of workers who recycled e-waste. A dose-effect relationship was found between the gene expression and exposure duration of e-waste (solid line box). The degree of redness and greenness represent induction and repression, respectively. (C): GEArray Expression Analysis Suite (SuperArray) was used to analyze the data clustered in the heat map. The black line indicates a fold-change in the gene expression of 1. The pink lines indicate significant fold-changes in the gene expression threshold. A boundary of 2 was set in a scatterplot analysis. Red and green dots showed the genes with a > 2-fold up-regulation and genes with a > 2-fold down-regulation, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Correlations between exposure time, serum Pb, MDA, GSH, SOD, Total PCBs and semen quality (R/*P*).

Group	MDA	GSH	SOD	Pb	Exposure duration	Total PCBs
Motility rate	−0.190/0.247	0.419/0.006	0.466/0.001	−0.408/0.093	−0.671/0.000	−0.382/0.018
Abnormality rate	0.445/0.005	−0.584/0.000	−0.580/0.000	0.389/0.111	0.695/0.000	0.426/0.015
Total sperm count	−0.477/0.001	0.604/0.000	0.612/0.000	−0.596/0.025	−0.583/0.001	−0.338/0.022
Exposure time	0.349/0.014	−0.476/0.001	−0.536/0.000	0.750/0.000		
Pb	0.489/0.013	−0.387/0.042	−0.570/0.002		0.750/0.000	
Total PCBs	0.195/0.152	−0.105/0.223	−0.251/0.183	0.217/0.161	0.693/0.000	

Table 5
Multivariate, logistic regression (backward elimination; α = 0.1) analysis of risk factors for sperm motility rate, abnormality rate and total sperm count (n = 267).

Variable	Exposure duration[a]		MDA[b]		GSH[b]		SOD[b]		Pb[b]		Total PCBs[b]	
	OR[c]	95% CI[d]	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Motility rate	0.4**	0.2–0.7	0.5*	0.4–1.3	0.9	0.6–1.4	1.0	0.9–1.1	0.4*	0.3–1.1	0.4*	0.3–1.0
Abnormality rate	1.9*	1.1–3.3	2.3*	1.1–4.9	0.8	0.5–1.3	1.0	0.9–1.1	0.9	0.8–1.1	2.0*	1.5–2.9
Total sperm count	0.5**	0.3–0.8	1.0	0.7–1.5	0.9	0.6–1.3	1.0	0.9–1.1	1.0	0.9–1.1	0.4	0.1–2.9

* $P < 0.05$.
** $P < 0.01$.
[a] Never involved in the e-waste recycling = 0, involved in the e-waste recycling = 1.
[b] The level of each variable ≤ mean level = 0, > mean level = 1.
[c] OR (odds ratio).
[d] CI (confidence interval).

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Declaration of interests

The authors declare they have no actual or potential competing financial interests.

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