Reproductive Toxicology 29 (2010) 184–190

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Chronic boron exposure and human semen parameters

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ARTICLE INFO

*Article history:*
Received 16 July 2009
Received in revised form
24 September 2009
Accepted 2 November 2009
Available online 3 December 2009

*Keywords:*
Boron
Borates
Boric acid
Semen
Sperm
Occupational health
Reproduction

ABSTRACT

Boron found as borates in soil, food, and water has important industrial and medical applications. A panel reviewing NTP reproductive toxicants identified boric acid as high priority for occupational studies to determine safe *versus* adverse reproductive effects. To address this, we collected boron exposure/dose measures in workplace inhalable dust, dietary food/fluids, blood, semen, and urine from boron workers and two comparison worker groups ($n = 192$) over three months and determined correlations between boron and semen parameters (total sperm count, sperm concentration, motility, morphology, DNA breakage, apoptosis and aneuploidy). Blood boron averaged 499.2 ppb for boron workers, 96.1 and 47.9 ppb for workers from high and low environmental boron areas ($p < 0.0001$). Boron concentrated in seminal fluid. No significant correlations were found between blood or urine boron and adverse semen parameters. Exposures did not reach those causing adverse effects published in animal toxicology work but exceeded those previously published for boron occupational groups.

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## 1. Introduction

Boron in the form of borates is distributed throughout the earth in soil, food, and water [1,2]. Borates have many industrial and medical applications, for example, in insulation materials, fiberglass, fire retardants, textiles, boro-silicate glass, adhesives, detergents/soaps/bleaches, cosmetics, pesticides, soldering and welding fluxes, and neutron capture cancer therapy. The element boron is essential for plants and is found in fertilizers [3]. It is likely an essential human nutrient as well [4], and can be found in some vitamin supplements. *In vitro* studies have shown that boron in the form of boric acid, phenylboronic acid, and calcium fructoborate targets proliferative properties of breast and prostate cancer cell lines [5–7]. Demand for this important element is increasing but evidence for safe *versus* adverse workplace or environmental exposure for human reproductive health is limited.

Boric acid was ranked as one of the four top chemicals in need of further field study by a consensus panel convened to review workplace reproductive toxicants [8]. The consensus panel ranked 43 National Toxicology Program (NTP) reproductive toxicants according to animal toxicology data, potential numbers of workers exposed in the USA, and epidemiologic data. Chemicals were ranked as high priority if animal toxicology data indicated a LOAEL of <250 mg/kg-d for reproductive endpoints in NTP Reproductive Assessment by Continuous Breeding (RACB) studies. Chemicals were ranked as high priority if production data indicated >100,000 workers potentially exposed based on NIOSH National Occupational Exposure Survey data and production of >500,000 tons based on the Hazardous Substance Database and USEPA High Production Volume Database. If there was little epidemiologic evidence for safe *versus* toxic effects in exposed workers, chemicals were rated as high priority. At the time of the consensus panel review, few epidemiologic studies had examined human reproductive health related to environmental or workplace borates and these studies had reported both no adverse effects [9,10] and testicular atrophy and sterility [11].

To address the question of whether exposures to boron are associated with human reproductive effects, we undertook a study of reproductive health in male boron miners and processing plant workers and comparison groups of men not working in the boron industry in Kuandian County, PR China. Males were the focus because animal toxicology data indicated male reproductive system sensitivity at lower doses relative to female [11]. Kuandian County was chosen because it contained geographic areas of both low and high environmental boron with boron industry worksites located in the areas of high environmental boron, plus desire of

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0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.11.003

local environmental and occupational professionals to evaluate the growing boron industry in their community.

This study was conducted in three phases. There were new recruitment efforts for each phase. Phase one (2002) involved interviews with 1187 men to learn about reproductive health, the boron industry, and the nature of work in this area of China [13]. Phase two (2003) involved detailed workplace and dietary exposure assessment with 69 men [14] in order to design and power the third and final research phase that involved sampling to encompass one entire cycle of spermatogenesis in order to ascertain boron effects on semen quality. Findings reported in this paper are based on data collected in 2004 during the third and final wave of this three phase research project.

## 2. Materials and methods

The research was reviewed and approved by both the University of California Institutional Review Board for the protection of human research subjects and the China National Environmental Monitoring Station Human Subjects Review Board. All study subjects gave informed consent prior to participation in the research.

### 2.1. Study subjects

Eligibility criteria were based on age (18–40 years), employment at the same worksite for at least the previous year, not currently under treatment for chronic disease, and no history of vasectomy. Boron workers ($n = 74$) were enrolled at five local boron industry sites. Two comparison groups were enrolled concurrently. A comparison control group ($n = 70$) was enrolled from a region with very little boron in ground water and soil ~30 miles away but close enough to share similar customs to the boron workers. A second comparison group ($n = 63$) was enrolled to assess effects of environmental exposure to boron through food and water due to living in the area of boron industry with high environmental boron but not working in the boron industry. This group is referred to as the "community comparison" group. Participation rates approached 95% and did not vary significantly across exposure groups.

### 2.2. Data collection

Interview data was collected using a 51 item questionnaire guide developed during phase one of the study in collaboration with a Community Advisory Board familiar with the local industry and customs. Inter-rater reliability of this instrument demonstrated an error rate of less than 0.8% as described by Chang et al. [13]. Consistency of data reported by subjects over the repeated sampling interval was greater than 95%. Domains included work, general health, reproductive health, diet, and lifestyle.

Boron exposure through inhalable dust was assessed using full workshift breathing zone air samples collected using Institute of Medicine (IOM) inhalable particulate mass (IPM) lapel filter cassettes and personal air monitoring pumps (PCXR series, SKC, Inc., Pennsylvania). Each pump was calibrated pre- and post-shift using a DryCal DC-Lite primary flow meter (BIOS, New Jersey). The flow rate was 2.0 L/min. The 25-mm mixed cellulose ester filters were assayed after weighing the cassette inserts then digested using heat and acid.

Boron exposure through food and fluids was assessed by collecting 24-h duplicate food and fluid intakes for all men during the exposure assessment phase in 2003 and a subset of volunteers (15 per exposure group) in 2004. A composite of total daily exposure was generated by adding exposure through workplace inhalable dust, food, and fluid intake and this was shown to be highly correlated with post-workshift urine [14]. Post-workshift urine was then used to predict total exposure for the study groups as described by Xing et al. [14].

Biologic markers of boron dose were measured in blood, urine, and semen. A research field laboratory was established in the local Kuandian County hospital for collection and processing of biological specimens. Sampling was identical for all exposure groups and included a blood specimen and collecting semen and post-workshift urine samples on three separate occasions (once a month spanning approximately three months). Collection of biologic samples was coordinated with same day inhalable workplace dust measurement. One exception was that men from the area of low environmental boron did not wear personal air samplers during workshifts because phase two data had shown no appreciable amount of boron in airborne dust in the area of low environmental boron.

Exposure was calculated in terms of elemental boron because borates dissociate in the body at physiological pH to boric acid [$B(OH)_3$] and borate [$B(OH)_4^-$] which have similar effects when dose is calculated as elemental boron [15]. Dust, dietary intakes of food and fluids were analyzed by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry) and biologic samples were analyzed by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) all at the National Research Center of Geo-analysis, Chinese Academy of Geological Sciences, Beijing, China. For all dust and biologic samples, a total of 10% field blanks (with a minimum of 2) for each day of sampling were collected and analyzed. Creatinine adjustment was used for post-workshift urine boron concentration [16].

### 2.3. Conventional semen parameters

Semen samples were collected at the Kuandian County hospital in a private room located adjacent to the research field laboratory. This allowed analysis of all semen samples within 60 min of sample production. Participants were asked to abstain from ejaculation for at least two days prior to providing research semen specimens. Polypropylene semen collection containers, storage vials, and pipettes were used throughout (Fisher Scientific, Pittsburgh, PA) that had been tested and found free of appreciable boron by West Coast Analytical Laboratory, Santa Fe Springs, CA. Two researchers who were trained in semen analysis laboratory techniques and were blinded as to subject exposure group performed the initial sample analyses and processed remaining sample for storage and transfer to the UCLA laboratory for sperm morphology and DNA integrity assays.

Each sample was allowed to liquefy at room temperature and routine semen analysis was performed according to standard procedures for appearance, viscosity, pH, and initial microscopic exam as described in the WHO guidelines [17]. Hamilton-Thorne Biosciences IVOS (Beverly, MA) was utilized for counting sperm and assessing motility parameters according to version 12.1 protocols. Standards for sperm count quality control were purchased from Fertility Solutions, Cleveland, OH. For sperm morphology, 10 μL of semen was smeared onto slides, fixed in 95% ethanol 10 min, and stained with Hema3® stain (Fischer Scientific). Slides were scored by a single technician who had completed an American Society of Andrology Sperm Morphology Laboratory Training. Test slides for quality control validation were from Fertility Solutions, Cleveland, OH. Strict morphology criteria [18] were used at 100× magnification.

### 2.4. Sperm DNA integrity assays

Sample size calculations from phase two of the research were used to determine an adequate subset number of subjects to be included for sperm DNA integrity assays due to the time intensive nature and expense of these laboratory procedures. All men in the boron worker group who had sufficient sample remaining after conventional semen analyses for the three DNA integrity assays and a randomly chosen subset of men with adequate sample remaining within each of the comparison groups were included ($n = 146$).

Sperm cells were evaluated for aneuploidy using sperm fluorescence *in situ* hybridization (FISH) adapted from Robbins et al. [19]. Direct labeled enumerator probes to the alpha satellite region for chromosome X (Xp11.1–q11.1) labeled with fluorophore SpectrumGreen, Y (Yp11.1–q11.1) labeled with SpectrumOrange, and 18 (18p11.1–q11.1) labeled with Spectrum Aqua were obtained from Vysis, Inc. (Downers Grove, IL.) as part of the AneuVysion™ DNA Probe Kit. Hybridized sperm were observed via fluorescence microscopy using a Zeiss Axiophot microscope with DAPI/FITC/Texas Red triple band pass filter set-up (61002) for simultaneous visualization of the different fluorochromes (Chroma Technology Corp. Brattleboro, VT). A single scorer systematically analyzed 8000 cells per sample according to strict scoring criteria [19]. Slides were coded so the scorer did not know if the subject was from boron worker group or a comparison group. Slides were chosen for scoring in random order.

Apoptotic DNA fragmentation was detected in 10,000 cells per sample using the Fluorescein FragEL™ DNA Fragmentation Detection Kit (Calbiochem, USA) and a flow cytometer equipped with a 488 nm argon ion laser light source (UCLA Flow Cytometry Core Facility, David Geffen School of Medicine). All specimens were batched and evaluated in a single run with controls: positive (DNase I) and negative ($dH_2O$ replacing TdT in the reaction mixture).

DNA strand breakage was detected using the COMET single cell gel assay as described by Young et al. [20]. A single scorer randomly selected and captured 50 cells from each of two replicate slides using the LAI Automated Comet Assay Analysis System (Loats Associates Inc., Westminster, MD). LAI COMET is an extended dynamic range, adaptive background correction fluorescent dye imaging system. Quantitative measurements reported include tail moment (product of migration distance and normalized intensity integrated over the tail length), moment arm (tail moment divided by the normalized integrated tail intensity to measure average distance of DNA migration within the tail), and percentage of DNA in the COMET tail (integrated tail intensity × 100 divided by the total integrated cell intensity). Specimens were prepared and scored in random order once the entire set of specimens had been collected. Positive controls (DNAase I) and negative controls (pooled standard known to have low breakage) were run with each set of samples.

### 2.5. Statistical analyses

Subjects were included in the statistical analyses if they had contributed three post-workshift urine samples over the follow-up period so as to document chronic boron exposure across a complete cycle of spermatogenesis [21,22]. During the follow-up period, eight men in the boron worker group, two men from the area of low environmental boron, and two from the community comparison group failed to provide three post-workshift urines. Additionally two men in the comparison group from the area around the mines and processing plants (community control

group) and one from the area of low environmental boron had changed employment within months of sampling so they were excluded. This gave a final sample size of 192 for statistical analyses of conventional semen parameters. All men in the boron worker group who had sufficient sample remaining after conventional semen analysis for the three DNA integrity assays and a randomly chosen subset of men with adequate sample remaining within each comparison group gave a final sample size of 146 for the statistical analyses of DNA integrity assays. In all cases, the semen sample provided at the end of the follow-up period was the one on which final study findings are based.

Variables were plotted and those that were highly skewed (i.e., some biological boron measures and semen parameters) were log transformed to normalize data prior to analysis. Chi squared tests and one-way ANOVA were used to evaluate relationships between the exposure groups on demographic, lifestyle and reproductive history variables. For variables found to be significantly different among the three exposure groups, two group *t*-tests were used to perform pair-wise comparisons utilizing multiple comparison corrections to retain an overall type I error rate of 5%.

Multiple linear regression models were constructed for each semen outcome using the boron levels in post-workshift urine as the predictor and examining the possible covariates of medications, chronic medical conditions, exposures to other known reproductive toxicants, history of reproductive problems, days abstinence prior to providing the semen sample (abstinence interval) and blood levels of other metals of interest (Mg, P, Se, Cr, As and Zn). Final models for each semen outcome included log urine boron and terms for age, smoking, alcohol, abstinence interval, and pesticide exposure. This limited set of covariates was included in the final models since the other covariates were not significantly associated with the semen outcomes and did not modify the effect of post-workshift urine boron. These procedures were repeated with log blood boron as the predictor and gave the same results overall. We report the Pearson correlation models with the single variable predictor "post-workshift urine boron" for the semen outcomes as well as the partial correlation between urine and the semen parameters in the final models. All statistical analyses were carried out in SAS [23] and R [24].

## 3. Results

Based on interview data, the three comparison groups did not differ significantly on general health status, history of infertility (defined as trying unsuccessfully for pregnancy more than one year), having a prior semen analysis, history of radiation or surgery or injury to the genital track, exposure to other known reproductive toxicants, use of contraception, years of marriage, spontaneous pregnancy loss, stillbirths, or birth defects in offspring ($p > 0.05$). The three groups did not differ on overall smoking habits or alcohol intake ($p > 0.05$). The comparison group from the area of low environmental boron reported more exposure to pesticides ($p = 0.07$). The community comparison group from the area with environmental boron reported lower levels of education at high school or above compared to the other groups but this did not reach statistical significance (Table 1). The means for self-reported abstinence interval were not statistically significantly different across the exposure groups ($p > 0.05$). The median abstinence interval was three days for each group, however, several men reported extended abstinence intervals that varied across the exposure groups. Evaluating the data with and without the men reporting extended abstinence intervals gave similar results overall. Because of the importance of abstinence interval to interpretation of semen parameters, especially total sperm count [25] it was included as a covariate in the final analyses.

The groups differed on boron measures in blood, semen, and post-workshift urine (Table 1). Boron workers had the highest levels of boron in blood, urine, and semen with the community comparison group falling between the boron worker group and the group from the area of low environmental boron. Boron workers were found to have the greatest total daily exposure to boron (composite of workplace dust, 24-h food and fluid intake) with 43% of their exposure estimated to be due to workplace dust. Total daily boron exposure was highly correlated with biological boron levels in blood, semen, and post-shift urine (Pearson correlation coefficient <0.0001 for each).

The values for conventional semen parameters in our study sample were consistent with those reported for similarly aged healthy men in China, some of which are lower than WHO reference values [17,26]. Each participant contributed three semen samples, one a month spanning approximately three months. Comparisons across the three samples showed them not to differ significantly on semen parameters ($p > 0.05$) except for morphology "percent normal forms" ($p = 0.04$) and "sperm head defects" ($p = 0.004$). Averaging the three samples and using the mean for statistical testing gave the same conclusions overall. Because the third sample was most representative of a complete cycle of spermatogenesis for which continuous exposure data was available and because published animal toxicology work had shown reversible testicular effects when boron exposures were reduced, analyses presented here are based on the third semen sample.

Parameters for total sperm count, sperm concentration, motility and morphology were not significantly different across the three

**Table 1**
Selected demographic and boron measures.

| | Boron worker ($n = 66$) | Community comparison ($n = 59$) | Control comparison ($n = 67$) | $p$-Value |
|---|---|---|---|---|
| *Demographics* | | | | |
| Age, years, mean (std dev) | 31.3 (4.4) | 30.0 (6.0) | 31.7 (5.2) | 0.18 |
| Range | 19–39 | 18–39 | 19–39 | |
| Education, $n$ (%) | | | | |
| Grade school | 12 (18.2) | 15 (25.4) | 15 (22.4) | |
| Middle school | 35 (53.0) | 33 (55.9) | 34 (50.8) | 0.68 |
| High school+ | 19 (28.8) | 11 (18.7) | 18 (26.8) | |
| Alcohol, $n$ = yes (%) | 33 (50.0) | 36 (62.0) | 33 (49.3) | 0.34 |
| Smoke, $n$ = yes (%) | 46 (69.7) | 34 (57.6) | 47 (70.2) | 0.25 |
| Pesticide exposure, $n$ = yes (%) | 13 (19.7) | 7 (11.9) | 19 (28.4) | 0.07 |
| *Boron measures* | | | | |
| Blood boron (ppb) | | | | |
| Mean (std dev) | 499.2 (790.6) | 96.1 (92.1) | 47.9 (24.1) | <0.0001 |
| Range | 20.4–3568.9 | 3.3–536.0 | 8.2–113.0 | |
| Semen boron (ppb) | | | | |
| Mean (std dev) | 785.6 (605.7) | 310.6 (245.3) | 214.0 (113.9) | <0.0001 |
| Range | 160.6–3542.0 | 87.6–1177.0 | 31.7–570.9 | |
| Post-shift urine | | | | |
| Boron mg/L[a] (std dev) | 16.7 (31.4) | 5.5 (15.6) | 2.0 (0.9) | 0.0001 |
| Range | 1.3–217.0 | 0.7–121.3 | 0.7–6.0 | |

std dev = standard deviation; $p$-values are based on ANOVA comparing the three exposure groups.

[a] Creatinine corrected.

**Table 2**
Sperm concentration, motility, and morphology parameters.

| | Boron worker ($n=66$)<br>Mean (std dev)<br>Range | Community comparison ($n=59$)<br>Mean (std dev)<br>Range | Control comparison ($n=67$)<br>Mean (std dev)<br>Range |
|---|---|---|---|
| Total sperm count ($\times 10^6$) | 223.4 (140.4)<br>6.7–602.9 | 219.8 (145.5)<br>4.1–622.7 | 224.8 (166.3)<br>18.4–730.7 |
| Sperm concentration ($\times 10^6$/mL) | 68.2 (39.4)<br>2.5–190.6 | 69.6 (48.5)<br>2.2–263.9 | 67.6 (42.4)<br>7.7–192.3 |
| Motile cells (%) | 51.3 (21.5)<br>3.0–87.0 | 50.9 (25.2)<br>0.0–92.0 | 49.1 (23.5)<br>0.0–93.0 |
| Straight line velocity (μm/s) | 46.3 (5.4)<br>33.9–57.1 | 44.1 (11.5)<br>0.0–61.9 | 45.6 (10.5)<br>0.0–60.6 |
| Curvilinear velocity (μm/s) | 89.1 (14.1)<br>63.3–130.6 | 85.4 (22.4)<br>0.0–122.5 | 87.0 (22.0)<br>0.0–139.0 |
| Linearity | 53.2 (6.1)<br>42.0–65.0 | 50.9 (11.2)<br>0.0–67.0 | 52.1 (10.9)<br>0.0–66.0 |
| Normal morphology (%) | 10.8 (6.5)<br>2.6–16.0 | 9.9 (4.1)<br>2.5–20.3 | 9.0 (4.0)<br>0.5–22.0 |
| Head defects (%) | 87.1 (6.1)<br>57.0–98.0 | 87.7 (4.7)<br>76.8–95.5 | 88.6 (4.5)<br>76.0–99.0 |
| Neck/mid-piece defects (%) | 15.7 (6.2)<br>5.0–30.5 | 16.2 (7.7)<br>3.5–35.0 | 16.6 (8.1)<br>5.0–53.0 |
| Tail defects (%) | 7.0 (3.9)<br>1.0–17.5 | 6.7 (5.0)<br>1.0–24.0 | 7.8 (5.4)<br>1.5–33.0 |
| Cytoplasmic droplets (%) | 0.8 (0.7)<br>0.0–2.5 | 0.8 (0.7)<br>0.0–2.5 | 0.8 (0.9)<br>0.0–4.5 |

boron exposure comparison groups (Table 2). Using regression modeling, exposure group did not predict any of the conventional semen parameters before or after controlling for age, abstinence interval, alcohol intake, smoking status, and pesticide exposure. However, continuous measures of boron in post-workshift urine and blood were correlated with percent normal morphology (Pearson correlation coefficient 0.15, $p=0.04$ for both urine and blood boron) but this did not remain statistically significant after controlling for age, abstinence interval, smoking, alcohol intake, pesticide exposure and Mg blood levels (Partial correlation coefficient 0.14, $p=0.06$ for post-workshift urine; 0.12, $p=0.11$ blood boron). No other significant correlations between boron levels and conventional semen parameters were found (Table 3). DNA strand breakage measured by the COMET assay and percent apoptotic cells measured by TUNEL assay were similar across the exposure groups and not correlated with boron levels in post-workshift urine or blood ($p>0.05$). Sperm aneuploidy and diploidy of chromosomes X, Y or 18 did not differ by exposure group or boron levels in post-workshift urine or blood ($p>0.05$). (Table 4). Aneuploidy and diploidy frequencies for our study group were consistent with those of same aged Chinese men reported by Shi and Martin [27].

**Table 3**
Pearson correlation between log boron in post-workshift urine and semen parameters before and after adjusting for age, abstinence interval, smoking, alcohol, and pesticide exposure.

| | Original scale | | After adjustment | |
|---|---|---|---|---|
| | Correlation | $p$-Value | Partial correlation | $p$-Value |
| Total sperm count | 0.02 | 0.74 | 0.04 | 0.58 |
| Sperm concentration | 0.04 | 0.60 | 0.04 | 0.60 |
| Percent motile sperm | 0.07 | 0.33 | 0.04 | 0.64 |
| Straight line velocity | 0.03 | 0.63 | 0.00 | 0.97 |
| Curvilinear velocity | 0.00 | 0.96 | 0.00 | 0.96 |
| Linearity | 0.11 | 0.14 | 0.05 | 0.55 |
| Normal morphology | 0.15 | **0.04** | 0.14[a] | 0.06[a] |
| Sperm head defects | −0.11 | 0.13 | −0.11 | 0.17 |
| Sperm neck and mid-piece defects | −0.01 | 0.17 | −0.09 | 0.26 |
| Tail defects | −0.02 | 0.76 | −0.03 | 0.74 |
| Cytoplasmic droplets | −0.03 | 0.71 | −0.01 | 0.90 |

0.04 is the significance as this is the $p$ value.
[a] Adjusted for age, abstinence interval, smoking, alcohol, pesticide exposure, and Mg

## 4. Discussion

Elemental boron is found at ~10 ppm in the earth's crust on average with some geographic regions deficient and a few with concentrations high enough to be of economic interest [1,2]. Most human exposures to boron occur through normal diet with average dietary intake around the world estimated at ~1.4 mg per day [28]. Certain populations may have higher exposures, for example, persons living on or near ore beds who ingest ground water containing borates and food grown in boron rich soils [9,29,30]. Workers in the boron mining and processing industry can be exposed through inhalation of borates in dusts generated at the workplace [15,31]. All of these potential sources of exposure (food, water, workplace dust) were evaluated in our study.

Borates, in general, have low toxicity related to general health [31,32] although animal toxicology studies suggest dose and time dependent reproductive system sensitivity [12,33,34]. For example, Linder et al. [35], Treinen and Chapin [36], Ku et al. [34] found exposure to high doses of boric acid resulted in testicular atrophy in rats. At lower doses, inhibited spermiation was identified as the earliest testicular lesion seen at 26 mg boron/kg-d (summarized by Ku and Chapin [37]). The researchers also found that testicular effects of boron at lower doses were reversible. Thus, to insure continual exposure over a full cycle of spermatogenesis in our human subjects, we only included men for whom we could confirm boron levels in three post-workshift urines collected once a month over three months of sampling. Urine is a good measure of total boron exposure because little boron accumulates in tissues and approximately 84–90% is subsequently excreted unchanged in

**Table 4**
DNA integrity assays.

| | Boron worker ($n = 63$)<br>Mean (std dev)<br>Range | Community comparison ($n = 39$)<br>Mean (std dev)<br>Range | Control comparison ($n = 44$)<br>Mean (std dev)<br>Range |
|---|---|---|---|
| COMET DNA in the tail | 6.97 (5.28)<br>0.70–24.78 | 8.19 (5.33)<br>1.50–25.51 | 7.70 (7.16)<br>0.53–43.47 |
| COMET moment | 1.53 (2.44)<br>0.02–16.18 | 1.81 (2.27)<br>0.13–11.61 | 1.91 (3.59)<br>0.01–19.27 |
| COMET moment arm | 15.72 (9.27)<br>1.97–64.26 | 17.43 (10.53)<br>8.49–67.51 | 16.30 (11.27)<br>1.45–67.64 |
| TUNEL % apoptotic cells | 18.91 (11.24)<br>2.57–69.39 | 15.05 (10.53)<br>2.82–54.49 | 21.30 (15.69)<br>6.10–87.18 |
| Total aneuploidy for chromosomes X, Y, 18/8000 cells scored | 56.1 (20.4)<br>23.00–116.00 | 47.4 (16.9)<br>20.00–91.00 | 62.9 (26.8)<br>13.00–145.00 |
| Total diploidy for chromosomes X, Y, 18/8000 cells scored | 14.6 (9.0)<br>3.00–56.00 | 12.5 (6.6)<br>4.00–31.00 | 16.6 (11.0)<br>2.00–59.00 |

COMET = LAI automated COMET (single cell gel) analysis (Loates Associates, Inc.); TUNEL = Flow Cytometry Fluorescein FragEL™ DNA Fragmentation Assay; aneuploidy and diploidy = sperm fluorescence *in situ* hybridization.

urine over a 72-h period [32,38,39]. Additionally, boron in creatinine adjusted post-workshift urine has been shown to be highly correlated with 24-h urine boron and total daily boron exposure [14].

We conducted a comprehensive assessment of semen quality indicators that included targeting human correlates of the toxic endpoints previously described in published animal toxicology literature related to boron. For example, an NTP reproductive assessment by continuous breeding study found that male rats treated with high doses of boric acid exhibited decreased sperm motility so we included both light microscope and computer aided measures for sperm motility. No significant association between boron exposure and sperm motility was found for the range of exposures in our human population that were lower than those causing toxic effects in the animal work. We had 90% power to detect a 20% difference between the exposure groups for the majority of motility parameters.

Animal toxicology data had demonstrated inhibited spermiation (release of sperm into the seminiferous tubule lumen) as an early testicular effect of boron exposure. We included measures of sperm morphology in our human study that would indicate an excess of residual cytoplasm [40] or other abnormal forms of sperm cells that might result from disturbed spermiation. A positive correlation between percent normal morphologic forms and levels of boron in post-workshift urine and blood was found but did not remain significant after controlling for age, smoking status, abstinence interval, alcohol intake, pesticide exposure, and Mg blood levels. This was consistent with the observation that increased boron in blood and urine correlated with decreases in specific types of defects (e.g., sperm head, neck and mid-piece defects) although specific categories of defects were not statistically significant. We controlled for blood levels of Mg in this analysis because other common metals found in raw ore of this region are Mg, Ca, Fe, Al and Mn in decreasing order of abundance. Local industry processing of ore for boron increases the concentration of Mg two- to threefold. Blood levels of Mg for our study population were within the normal clinical range for this element and ranged from 16.6 to 27.9 ppb. Mg levels in post-workshift urine and blood were correlated with fewer sperm neck and mid-piece abnormalities ($p < 0.01$). Normal sperm morphology is associated with fertility [17]. Whorton et al. [10] reported increased fertility for boron workers compared to the USA population using a Standardized Birth Ratio (SBR) although use of the SBR was subsequently challenged as inappropriate. We were unable to confirm increased fertility for boron workers when comparing them to non-boron workers from areas of low environmental boron or high environmental boron within our sample of 192 men. Additionally, increased fertility for boron workers compared to men from an area of low environmental boron was not found in the earlier questionnaire phase of this research project based on 1066 men who had been married for at least one year [13]. We measured fertility by questionnaire/interview including men who had been married greater than one year. Ascertaining fertility using historical questionnaire data collected from the male partner in couples can be problematic; however, social norms in this region of China, such as limited numbers of children and obtaining government approval prior to planning a pregnancy may improve recall of time to pregnancy.

Cell culture experiments by Ku and Chapin [37] led them to postulate that boric acid affects DNA synthesis activity of mitotic and meiotic germ cells leading to reduced leptotene spermatocyte/sertoli cell ratio. Trienen and Chapin [36] noted that viable germ cells were prematurely shed in boron exposed rats, a phenomenon also seen with colchicine and vinblastine that affect microtubules [41]. For evidence of perturbation of maturation of germ cells through mitosis and meiosis we included measures of aneuploidy and diploidy but found no association with exposure group or boron levels in post-workshift urine or blood in the range of boron exposure for our human population. The only effect noted was a lower ratio of Y to X bearing sperm in boron workers compared to men not working in the industry [42]. This finding of reduced Y:X sperm ratio was replicated in two phases of the research project, phase two data based on 60 boron workers and 9 controls ($p = 0.02$) and again in phase three based on 63 boron workers, 44 men from an area of low boron, and 39 men from an area with high environmental boron ($p < 0.0001$). The ratio of Y to X bearing sperm was not significantly correlated with any other conventional semen parameter (sperm concentration, motile cells, morphology, apoptosis, or DNA fragmentation measured by COMET assay). No statistically significant within group correlations were found for the biological boron measures and Y:X ratio.

Animal toxicology work found that blood boron concentrations approximately equilibrated to other tissues examined (kidney, liver, epididymis, and testes [36]), however, in our human subjects boron appeared to concentrate in seminal fluid averaging 1.6, 3.2, and 4.5 times that found in blood for the boron worker group, community comparison group, and low environmental boron group respectively. This may be a function of accumulation of boron in sexual accessory glands or the prostate as 95% of the ejaculate volume originates from these tissues [43]. This finding in the human is interesting given reports that boric acid inhibits the growth of

LNCaP prostate cancer tumors in nude mice [44] and inhibits LNCaP and DU-145 prostate cancer cell lines in culture [7].

There are limitations when comparing animal toxicology experiments to our human epidemiologic approach. Unlike animal experiments, in our epidemiologic study we could not manipulate exposure to borates. Boron exposures in animal work were controlled single agent exposures (usually boron in the form of boric acid). In our boron workers exposures were not single agent as there were lifestyle factors to consider and ore in this area contained multiple elements, some at relatively high concentrations (e.g., Mg). At the beginning of our five year study in Kuandian County, the boron mining and processing workers were more highly exposed to workplace dust compared to the final sampling in 2004 as we noted increasing use of respirators and engineering controls over the time span of the research. Still, compared to worker groups in the USA previously described in the published literature [45], the workers in this area of China had higher total daily exposures although even the most highly exposed group (average 0.6 mg boron/kg-d) fell below the level causing the earliest testicular lesions (mild inhibited spermiation) in rat studies at 26 mg boron/kg-d [34].

## 5. Conclusion

Boron workers in our study experienced chronic exposure to boron over one complete cycle of spermatogenesis. When compared to healthy working men living in an area of low environmental boron and healthy working men living near or on the boron ore beds but not employed in the boron industry, we found no adverse association between exposure group and conventional semen parameters (total sperm count, sperm concentration, motility and morphology) or sperm DNA integrity measures (aneuploidy, DNA strand breakage and apoptosis). We specifically looked in our human subjects for toxicologic endpoints reported in animal chronic dosing experiments but did not find evidence to suggest human sensitivity at the exposure levels encountered by our study population that averaged 42 mg boron per day (standard deviation 58 mg boron per day). We found lower Y:X ratios in sperm of boron workers compared to men not employed in the boron industry and that boron concentrated in seminal fluid compared to blood. The concentration of boron in seminal fluid is interesting in light of current investigations looking at inhibitory effects of boron related to prostate cancer.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

## Acknowledgments

This work was supported by RO1 OHO7575 from the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, and the UCLA Center for Occupational and Environmental Health. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the awarding agencies. The authors deeply acknowledge the assistance of the China National Environmental Monitoring Station and the Boron Epidemiology Research Group.

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