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Article

Associations of Urinary Trichloroacetic Acid Concentrations with Spermatozoa Apoptosis and DNA Damage in a Chinese Population

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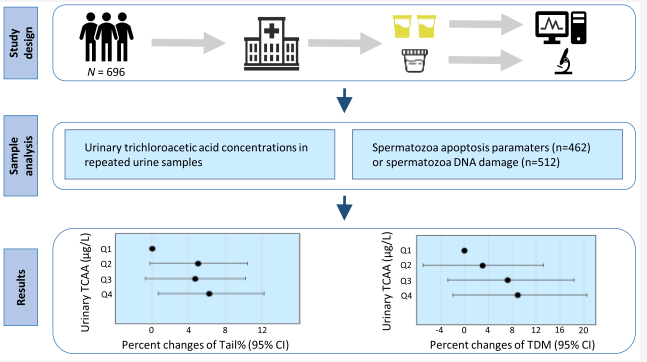
ABSTRACT: Exposure to trichloroacetic acid (TCAA) has been associated with impaired semen quality; however, its association with spermatozoa apoptosis and DNA damage remains unclear. We, therefore, collected single semen and repeated urine samples from male partners of couples attending a reproductive center, which were measured for spermatozoa apoptosis and DNA damage parameters and TCAA concentrations, respectively. Multivariable linear regression models were used to explore the associations between urinary TCAA concentrations and spermatozoa apoptosis (n = 462) and DNA damage parameters (n = 512). After adjusting for potential confounders, positive dose−response relationships were found between urinary TCAA concentrations and percentage of tail DNA (tail%) and tail-distributed moment (TDM) (both p for trend <0.10). Compared with men in the lowest tertile of urinary TCAA concentrations, men in the highest tertile had a greater tail% and TDM of 6.2% (95% CI: 0.7, 12.2%) and 8.9% (95% CI: −1.9, 20.5%), respectively. Urinary TCAA concentrations were unrelated to spermatozoa apoptosis parameters in a dose−response manner. However, urinary TCAA concentrations were positively associated with the percentage of Annexin V^+/PI^- spermatozoa (apoptotic cells), when urinary TCAA concentrations were modeled as continuous variables. Our results suggest that exposure to TCAA at concentrations in real-world scenarios may be associated with spermatozoa apoptosis and DNA damage.



KEYWORDS: *apoptosis, DNA damage, reproductive health, epidemiology, spermatozoa, trichloroacetic acid*

INTRODUCTION

Disinfection byproducts (DBPs) are widespread water contaminants formed when oxidizing disinfectants react with organic matter in raw water.[1] More than 700 DBPs have been identified to date, among which haloacetic acids (HAAs) are the leading species of nonvolatile DBPs.[2] Exposure to HAAs occurs mainly through ingestion of water. Consequently, the concentrations of trichloroacetic acid (TCAA) in urine, valid biomarkers that reflect ingestion of HAAs in chlorinated drinking water,[3] are detectable in more than three-quarters of adults, children, and pregnant women from many countries, including China.[4,5] Given the cytotoxicity, carcinogenicity, genotoxicity, and reproductive toxicity of HAAs,[6,7] the maximum contaminant level (MCL) for TCAA has been regulated to 100 μg/L by the Chinese drinking water standard.[8]

Toxicological studies have demonstrated the adverse effect of HAA exposure on the internal structure of testis and epididymis,[6] sperm morphology,[9] and sperm motility parameters.[10,11] In humans, several studies also reported associations between HAA exposure and reduced semen quality parameters, including sperm count, concentration, motility, and morphology.[12,13] In addition to these traditional semen quality parameters, several laboratory techniques used to evaluate spermatozoa apoptosis and DNA damage are strongly recommended because of their high repeatability and ability to measure different aspects of semen quality.[14] Accumulating evidence from in vivo and in vitro experiments shows that DBPs, including HAAs, can trigger genetic damage and apoptosis.[15−18] However, no human study has explored the associations of HAA exposure with spermatozoa apoptosis or DNA damage. Therefore, we explored the associations of exposure to HAAs with spermatozoa apoptosis and DNA damage in a Chinese population, using TCAA concentrations measured in repeated urine samples as internal exposure biomarkers.

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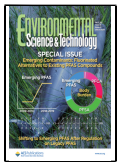

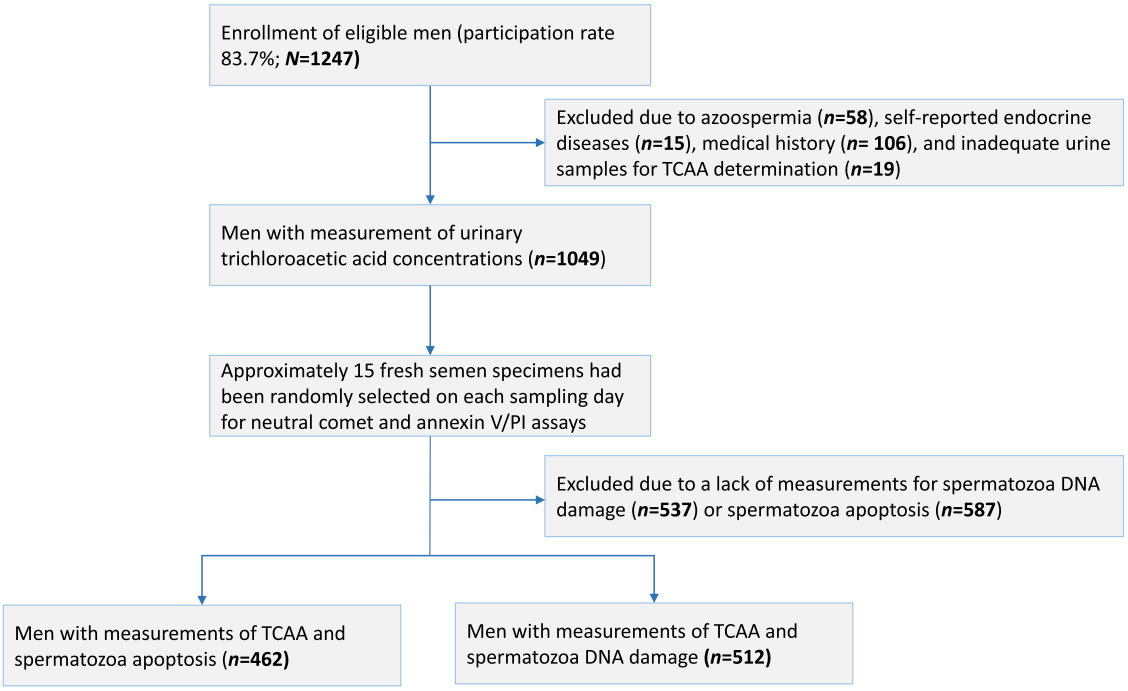

Figure 1. Flow chart for the study population.

MATERIALS AND METHODS

Participants. From March to June 2013, we recruited 1247 male partners of subfertile couples seeking semen analysis at the Reproductive Center of Tongji Hospital, Wuhan, China. Eligibility criteria have been previously described in detail.[19,20] We randomly selected about 15 fresh semen samples on each sampling day to conduct neutral comet and annexin V/PI assays within 1 h after sample collection, given that cryopreservation can induce spermatozoa apoptosis and DNA damage.[21,22] We excluded 121 men due to self-reported diseases (e.g., diabetes, epididymitis, vasectomy, orchiditis, testis injury, and adrenal disorder) that may impair semen quality, 58 men due to azoospermia, and 19 men due to lack of sufficient urine volume for TCAA determination (see Figure 1). We further excluded 587 and 537 men whose semen samples were not measured for spermatozoa apoptosis and DNA damage, respectively. Consequently, 462 and 512 men were included for the analyses of spermatozoa apoptosis and DNA integrity, respectively. The demographic characteristics of participants included in the current analysis were similar to that of the overall study population (Table S1). All men signed the informed consent. The research protocol was approved by the Ethics Committee of Tongji Medical College.

Questionnaire. Each participant completed a questionnaire, which collected data on demographic characteristics (e.g., age, BMI, race, income, education level, and marital status), lifestyle factors (e.g., smoking and alcoholic beverage intake), reproductive history (e.g., having ever fathered a pregnancy), and water-use activities. Nonsmokers were defined as individuals who did not smoke in the last three months, including men who never smoked and those who quit smoking more than 90 days. Nondrinkers were defined as those who consumed alcoholic beverages no more than once per week.[23]

Urine Collection and Determination. Each man provided two spot urine samples at different time points (at least 2 h apart) in a polypropylene container on the day of his clinic visit.[20] We froze these samples at −40 °C until laboratory determination. Urinary TCAA concentrations were determined using the established liquid−liquid extraction gas-chromatographic method (LLE-GC).[24] In brief, 10 mL of urine containing 1,2-dipropyl bromide was acidified with sulfuric acid and extracted using methyl-tert-butyl-ether. TCAA in these samples was converted to its methyl ester by reacting with acidic methanol and then determined by the Agilent Technologies 6890 N gas chromatograph (GC) with an electron capture detector. For quality control, one boiled spring-water sample and two pooled controls spiked with TCAA were analyzed along with every 30−40 samples. The analyte in boiled spring-water samples was all below the limits of detection (LOD; 0.5 μg/L for TCAA). The spiked recoveries for TCAA ranged from 90 to 115%. Urinary creatinine (Cr) concentrations were also determined to correct for urinary dilution using a clinical analyzer from Mindray Medical International Ltd., China.[20]

Semen Collection and Analysis. Each man provided a semen sample by masturbation into sterile polypropylene specimen cups in a specialized room after 2−7 days of abstinence. Neutral comet and annexin V/PI assays were separately conducted to measure spermatozoa apoptosis and DNA damage parameters in fresh semen samples (within 1 h after collection), as described in our previous study.[19] In brief, tail length (μm), percentage of tail DNA (tail%), and tail-distributed moment (TDM) (μm) were evaluated using the Comet Assay Software Project Lab (CASP, 2004) image analysis system. The percentage of apoptotic cells (Annexin V^+/PI^- spermatozoa), viable cells (Annexin V^-/PI^- spermatozoa), and dead cells (PI^+ spermatozoa) were measured by flow cytometry. Technicians were blind to individual information. Traditional semen quality parameters [e.g., sperm concentration, progressive motility (PR), nonprogressive motility (NP), and semen volume] were analyzed by professional technicians at the Reproductive Center of Tongji Hospital according to the fifth edition of the World Health

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Table 1. Characteristics of the Study Population [mean ± SD or *n* (%)]

characteristic	men measured for spermatozoa apoptosis parameters (*n* = 462)	men measured for DNA damage parameters (*n* = 512)	men measured for either spermatozoa apoptosis or DNA damage parameters (*n* = 696)
age, years	31.8 ± 5.2	32.1 ± 5.2	31.9 ± 5.2
BMI, kg/m^2	23.3 ± 3.1	23.2 ± 3.0	23.3 ± 3.1
race			
Han	448 (97.0)	497 (97.1)	678 (97.4)
other	14 (3.0)	15 (2.9)	18 (2.6)
having ever fathered a pregnancy			
yes	179 (38.7)	188 (36.7)	265 (38.1)
no	281 (60.8)	322 (62.9)	427 (61.3)
missing	2 (0.5)	2 (0.4)	4 (0.6)
abstinence time, days			
<3	48 (10.4)	54 (10.5)	74 (10.7)
3−5	307 (66.5)	341 (66.6)	461 (66.2)
>5	106 (22.9)	117 (22.9)	160 (23.0)
missing	1 (0.2)	0 (0.0)	1 (0.1)
education level			
less than high school	178 (38.5)	202 (39.5)	271 (38.9)
high school and above	280 (60.6)	305 (59.6)	419 (60.2)
missing	4 (0.9)	5 (0.9)	6 (0.9)
smoking history			
never	187 (40.5)	203 (39.6)	278 (39.9)
former	39 (8.4)	60 (11.8)	73 (10.5)
current	236 (51.1)	249 (48.6)	345 (49.6)
alcohol consumption			
yes	179 (38.7)	197 (38.5)	277 (39.8)
no	283 (61.3)	315 (61.5)	419 (60.2)
income, RMB yuan/month			
≤3000	200 (43.3)	224 (43.7)	305 (43.8)
3001−6000	166 (35.9)	191 (37.3)	258 (37.1)
≥6001	95 (20.6)	96 (18.8)	131 (18.8)
missing	1 (0.2)	1 (0.2)	2 (0.3)

Organization (WHO) laboratory manual,[25] as described previously.[20] We calculated total sperm motility (PR + NR) and total sperm count (semen volume × sperm concentration).

Statistical Analysis. Descriptive statistics were conducted for participants' demographic characteristics and the distribution of exposure and outcome measurements. Concentrations of TCAA lower than LOD were replaced with the LOD divided by the square root of 2.[26] The variability of repeated urinary TCAA concentrations was evaluated by calculating the intraday intraclass correlation coefficient (ICC), which is the ratio of between-person variance to the total variance.[27]

TCAA concentrations in repeated within-individual samples were $\log_{10}$-transformed and then averaged in all subsequent analyses. For participants who had a single measurement of TCAA (13.4%), we directly used their $\log_{10}$-transformed values. Multivariable linear regression models were conducted to assess the associations between urinary TCAA concentrations and spermatozoa apoptosis and DNA damage parameters. We divided within-individual average TCAA concentrations into quartiles to evaluate potential dose−response relationships. Tests for trend across quartiles were conducted by modeling the quartiles of TCAA concentrations as ordinal categorical variables using integer values. We also included urinary TCAA concentrations as continuous variables in multivariable linear regression models to explore potential linear associations. All spermatozoa apoptosis and DNA damage parameters were $\log_{10}$-transformed to improve the normality assumption of the linear models, except for the percentage of Annexin V^-/PI^- spermatozoa because of its approximately normal distribution. We reported percent changes for the acquired estimates and 95% CIs on the $\log_{10}$ scale using the formula: $100\% \times (10^{\text{estimate}}\text{-}1)$.

Covariates were selected based on biological and statistical considerations. Urinary creatinine concentrations were included in the regression models as continuous variables, which has been demonstrated to be a reliable method to account for urine dilution.[28] Other covariates with p values <0.2 in their relationship with at least one outcome measure in bivariate analyses were considered for inclusion. Covariates with a *p*-value >0.15 for all outcome measures were removed from the final models. To keep consistency, all final models included the same set of covariates, including urinary creatinine concentrations (arithmetic mean values), BMI (continuous), age (continuous), race (Han or others), and abstinence time (continuous), having ever fathered a pregnancy (yes vs no), smoking status (current, former, and never), and income (≤3000, 3001−6000, and ≥6001).

Since cigarette smoking may affect spermatozoa apoptosis and DNA damage,[29−31] we conducted stratified analyses according to smoking status in the past three months (nonsmokers vs current smokers) to assess its potential effect modification. Stratified analyses were also conducted according

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Table 2. Distribution and Intraclass Correlation Coefficients (ICCs) of TCAA Concentrations in Repeated Spot Urine Samples[a]

TCAA	the first urine sample			the second urine sample			variance component		
	%>LOD[c]	median	interquartile	%>LOD[c]	median	interquartile	interindividual (%)	intraindividual (%)	ICCs
Participants Measured for Spermatozoa Apoptosis Parameters (n = 462)									
unadjusted (μg/L)	96.8	2.38	1.38−4.17	91.3	2.08	1.05−3.65	12.1 (79.2%)	3.2 (20.8%)	0.79
Cr-adjusted[b] (μg/g creatinine)	—	1.82	1.11−3.01	—	1.79	1.06−3.49	10.0 (68.8%)	4.5 (31.2%)	0.69
Participants Measured for DNA Damage Parameters (n = 512)									
unadjusted (μg/L)	97.3	2.61	1.35−4.63	92.6	2.20	1.07−4.19	29.7 (86.4%)	4.7 (13.6%)	0.86
Cr-adjusted[b] (μg/g creatinine)	—	1.89	1.15−3.68	—	1.90	1.08−3.60	17.1 (82.3%)	3.1 (17.7%)	0.82
Participants Measured for Either Spermatozoa Apoptosis or DNA Damage Parameters (n = 696)									
unadjusted (μg/L)	97.1	2.44	1.37−4.45	91.5	2.10	1.01−3.89	23.3 (83.6%)	4.6 (16.4%)	0.84
Cr-adjusted[b] (μg/g creatinine)	—	1.89	1.14−3.39	—	1.89	1.07−3.58	14.1 (74.3%)	4.9 (25.7%)	0.74

[a]Abbreviations: TCAA, trichloroacetic acid; Cr, creatinine, LOD, limits of detection; ICCs, intraclass correlation coefficients. [b]Creatinine-adjusted urinary TCAA concentrations (μg/g) were calculated by dividing the crude target compound concentrations (μg/L) by creatinine concentrations (g/L) to correct for urine dilution. [c]The LOD for urinary TCAA concentrations: 0.5 μg/L.

Table 3. Distributions of Spermatozoa Apoptosis and DNA Damage Parameters

variables	arithmetic mean	geometric mean	percentile			range
			25th	50th	75th	
Spermatozoa Apoptosis (n = 462)						
annexin V^-/PI^- spermatozoa (%)	71.4	69.6	64.4	74.5	81.9	20.5−95.2
annexin V^+/PI^- spermatozoa (%)	15.7	12.5	8.2	12.6	19.5	0.2−76.3
PI^+ spermatozoa (%)	12.9	10.1	6.4	10.1	16.5	1.0−72.8
Spermatozoa DNA Damage Parameters (n = 512)						
percentage of tail DNA (%)	35.2	34.5	30.0	33.8	38.9	19.5−64.9
tail length (μm)	36.2	35.1	29.7	34.2	39.9	21.2−90.0
tail-distributed moment (μm)	15.4	14.2	10.8	13.6	17.8	5.8−53.7

to semen quality (normal vs abnormal) to assess if our findings persisted among participants who had normal sperm quality parameters. To keep consistency with our previous studies,[32,33] men whose all four parameters were equal to or greater than the fifth edition of WHO reference values (total count: 39 million; concentration:15 million/mL; progressive sperm motility: 32% motile sperm; and total motility: 40% motile sperm) were categorized as normal subjects.[25] To assess the robustness of creatinine-adjusted models, we (a) reanalyzed the associations between urinary TCAA concentrations and spermatozoa apoptosis and DNA damage parameters by excluding participants who had abnormal urinary creatinine (i.e., <0.3 and >3.0 g/L) and (b) used the "covariate-adjusted standardization plus covariate adjustment" method developed by O'Brien[34] to correct for urinary dilution. Data analyses were performed using the Statistical Product and Service Solutions, version 23.0 (IBM Corporation, Armonk, NY).

RESULTS

Population's Characteristics. We included 696 participants with either measurement of spermatozoa apoptosis or DNA damage parameters (Table 1). Participants were primarily of Han ethnicity (97.4%) with a mean age of 31.9 (±5.2) years and BMI of 23.3 (±3.1) kg/m^2. More than one-third of men (n = 265) had ever fathered a pregnancy. Nearly 60% of the volunteers (n = 419) graduated from high school or above, 49.6% (n = 345) had smoking habits at recruitment, and 39.8% (n = 277) consumed alcoholic beverages more than once per week.

Distribution of Urinary TCAA Concentrations. TCAA concentrations were detectable in >90% of the samples (Table 2). Among 696 participants with either measurement of spermatozoa apoptosis or DNA damage parameters, their median concentration of creatinine-adjusted TCAA was 1.89 μg/g creatinine (Table 2), which was similar to that of the overall study population (Table S1). The ICC of creatinine-adjusted TCAA concentrations in repeated urine samples was 0.74, indicating high reproducibility.

Distribution of Spermatozoa Apoptosis and DNA Damage Parameters. Among 462 men who were tested for the apoptosis assay, the median percentages of Annexin V^-/PI^- (viable cells), Annexin V^+/PI^- (apoptotic cells), and Annexin PI^+ spermatozoa (dead cells) were 74.5, 12.6, and 10.1%, respectively (Table 3). Among 512 men whose semen samples were tested for comet assay, the median values of tail %, tail length, and TDM were 33.8%, 34.2 μm, and 13.6 μm, respectively (Table 3).

Associations of Urinary TCAA with Spermatozoa Apoptosis and DNA Damage Parameters. Urinary TCAA concentrations were unrelated to spermatozoa apoptosis parameters in a dose−response manner (Table 4). In the fully adjusted models, however, urinary TCAA concentrations were positively associated with the percentage of Annexin V^+/PI^- spermatozoa (p = 0.04), when urinary TCAA concentrations were modeled as continuous variables. Additionally, we observed positive dose−response relationships between urinary TCAA concentrations and tail% and TDM (both p for trend <0.10). After adjusting for urinary creatinine concentrations and confounders, men in the highest tertile of urinary TCAA concentrations had a greater tail% and TDM of 6.2% (95% CI: 0.7, 12.2%) and 8.9% (95% CI:−1.9, 20.5%), respectively, compared with the men in the lowest tertile (Table 5). When we included TCAA concentrations as continuous variables, the positive association between urinary

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Table 4. Regression Coefficients or Percent Changes (95% CI) of Spermatozoa Apoptosis Parameters in Relation to Urinary TCAA Concentrations (n = 462)[a]

TCAA	annexin V$^-$/PI$^-$ spermatozoa	annexin V$^+$/PI$^-$ spermatozoa[b]	PI$^+$ spermatozoa[b]
	Model 1[c]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	0.3 (−3.4, 4.0)	4.2% (−14.8, 24.5%)	5.2% (−14.6, 27.1%)
Q3	−0.1 (−3.8, 3.5)	−2.1% (−21.9, 17.2%)	5.7% (−14, 27.6%)
Q4	−0.7 (−4.4, 3.0)	5.9% (−13.0, 26.5%)	11.2% (−8.4, 34.3%)
p for trend	0.67	0.70	0.28
	Continuous Analysis		
$\log_{10}$-transformed TCAA	−1.1 (−4.8, 2.5)	9.8% (−8.8, 31.2%)	8.8% (−10.8, 31.2%)
p values	0.54	0.30	0.38
	Mode 2[d]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	−0.2 (−4.0, 3.5)	6.7% (−12.5, 27.9%)	5.7% (−14.3, 27.9%)
Q3	−0.9 (−4.8, 2.9)	1.6% (−18.3, 22.5%)	6.7% (−14.0, 29.4%)
Q4	−1.8 (−5.8, 2.2)	11.7% (−8.4, 35.5%)	12.5% (−8.9, 37.7%)
p for trend	0.33	0.36	0.27
	Continuous Analysis		
$\log_{10}$-transformed TCAA	−2.4 (−6.4, 1.6)	17.3% (−3.4, 42.2%)	9.7% (−11.7, 34.4%)
p values	0.24	0.11	0.37
	Model 3[e]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	0.1 (−3.6, 3.9)	10.9% (−8.4, 33.4%)	−0.7% (−21.1, 19.7%)
Q3	−1.0 (−4.9, 2.9)	6.2% (−13.8, 28.2%)	4.0% (−16.1, 25.9%)
Q4	−1.8 (−5.9, 2.2)	16.4% (−4.5, 41.6%)	7.9% (−13.2, 31.5%)
p for trend	0.31	0.20	0.39
continuous analysis			
$\log_{10}$-transformed TCAA	−2.4 (−6.5, 1.6)	22.3% (0.6, 48.8%)	5.5% (−15.6, 28.6%)
p values	0.24	0.04	0.60

[a]Abbreviations: TCAA, trichloroacetic acid. [b]Since urinary TCAA concentrations, Annexin V$^+$/PI- spermatozoa, and PI$^+$ spermatozoa were $\log_{10}$-transformed, the estimation for these parameters were back-transformed {100% × (10^{estimate}-1)} to obtain percent change. [c]Crude model. [d]Adjusted for creatinine (continuous). [e]Adjusted for creatinine (continuous), BMI (continuous), age (continuous), abstinence time (continuous), race (Han vs others), having ever fathered a pregnancy (yes vs no), income (≤3000, 3001−6000 vs ≥6001), and smoking status (current and former vs never-smoker).

Table 5. Percent Changes (95% CI) of Spermatozoa DNA Damage Parameters in Relation to Urinary TCAA Concentrations (n = 512)[a]

TCAA	tail%[b]	tail length[b]	TDM[b]
	Model 1[c]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	5.2% (0.2, 10.7%)	−0.2% (−6.2, 5.4%)	4.5% (−5.2, 14.8%)
Q3	5.0% (−0.2, 10.4%)	4.7% (−0.9, 10.9%)	8.9% (−1.2, 19.7%)
Q4	6.7% (1.4, 12.2%)	4.2% (−1.6, 10.4%)	11.4% (1.4, 22.5%)
p for trend	0.02	0.06	0.02
	Continuous Analysis		
$\log_{10}$-transformed TCAA	5.0% (0.4, 9.7%)	2.2% (−2.8, 7.5%)	7.9% (−0.7, 17.3%)
p values	0.03	0.38	0.07
	Model 2[d]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	5.4% (0.2, 11.2%)	−0.9% (−6.9, 5.0%)	4.0% (−5.9, 14.6%)
Q3	5.2% (−0.2, 10.9%)	4.0% (−2.1, 10.4%)	8.4% (−1.9, 19.4%)
Q4	6.9% (1.4, 13.0%)	3.0% (−3.0, 9.6%)	10.7% (0.0, 22.7%)
p for trend	0.03	0.15	0.04
	Continuous Analysis		
$\log_{10}$-transformed TCAA	5.2% (0.3, 10.3%)	0.9% (−4.6, 6.5%)	6.9% (−2.3, 16.9%)
p values	0.04	0.75	0.14
	Model 3[e]		
	Quartile Analysis		
Q1	reference	reference	reference
Q2	5.0% (−0.2, 10.4%)	−1.6% (−7.6, 4.5%)	3.0% (−6.9, 13.2%)
Q3	4.7% (−0.7, 10.2%)	3.3% (−2.8, 9.6%)	7.2% (−2.8, 18.3%)
Q4	6.2% (0.7, 12.2%)	1.9% (−4.5, 8.6%)	8.9% (−1.9, 20.5%)
p for trend	0.04	0.28	0.08
	Continuous Analysis		
$\log_{10}$-transformed TCAA	4.7% (−0.2%, 9.8%)	−0.2% (−5.8, 5.5%)	5.3% (−4.0, 15.2%)
p values	0.06	0.95	0.26

[a]Abbreviations: TCAA, trichloroacetic acid; tail%, percentage of tail DNA; TDM, tail-distributed moment. [b]Since urinary TCAA concentrations, tail%, tail length, and TDM were $\log_{10}$-transformed, the estimations for these parameters were back-transformed {100% × (10^{estimate}-1)} to obtain percent change. [c]Crude model. [d]Adjusted for creatinine (continuous). [e]Adjusted for creatinine (continuous), BMI (continuous), age (continuous), abstinence time (continuous), race (Han vs others), having ever fathered a pregnancy (yes vs no), income (≤3000, 3001−6000 vs ≥6001), and smoking status (current and former vs never-smoker).

TCAA concentrations and tail% was attenuated but remained suggestively associated (p = 0.06).

Stratified analyses showed that the positive associations between urinary TCAA concentrations and tail% and TDM were slightly stronger among nonsmokers (Table S2) and men with normal semen quality (Table S3). Additionally, we found that urinary TCAA concentrations were positively associated with the percentage of dead spermatozoa (PI$^+$ spermatozoa) among nonsmokers (Table S4) and negatively associated with the percentage of viable cells (Annexin V$^-$/PI$^-$ spermatozoa) among nonsmokers (Table S4) and men with normal semen quality (Table S5).

The positive associations between urinary TCAA concentrations and tail% and TDM were substantially unchanged when we excluded men whose urinary creatinine concentrations were <0.3 or >3.0 g/L (Table S6). When we used the "covariate-adjusted standardization plus covariate adjustment" method to correct for urinary dilution (Table S7), these

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positive associations were slightly attenuated. Additionally, we found a positive dose−response relationship between urinary TCAA concentrations and the percentage of apoptotic spermatozoa (Annexin V^+/PI^-) in the "covariate-adjusted standardization plus covariate adjustment" models (p for trend = 0.04; Table S7).

DISCUSSION

Among 696 Chinese men attending an infertility clinic, we found positive dose−response relationships between urinary TCAA concentrations and tail% and TDM of spermatozoa. These associations were stronger among nonsmokers and men who had normal semen quality. Urinary TCAA concentrations were unrelated to spermatozoa apoptosis parameters in a dose−response manner. Notwithstanding, we found a positive association between urinary TCAA concentrations and the percentage of apoptotic spermatozoa when urinary TCAA concentrations were modeled as continuous variables and when we used the "covariate-adjusted standardization plus covariate adjustment" models to correct for urinary dilution.

A substantial body of literature has demonstrated the cytotoxicity and genotoxicity of HAAs, as manifested by induced cell stress, disrupted cell cycle and proliferation, DNA damage, and chromosomal or gene mutations from both in vivo and in vitro studies.[7,17,35−39] In a relevant study, Ali and colleagues observed that HAA compounds caused head and tail DNA damage both in human lymphocytes and germ cells using the comet assay.[18] To our knowledge, our study is the first to explore the associations between HAA exposure and human spermatozoa apoptosis and DNA damage, expanding our previous findings showing that urinary TCAA concentrations were inversely associated with traditional sperm quality parameters (e.g., sperm count, concentration, motility, and morphology) among men recruited from the same reproductive center in 2008[40] and 2011−2012.[12] Previous studies have shown that spermatozoa apoptosis and DNA damage parameters are highly reproducible and have the strength of providing additional aspects of male reproductive health and fertility.[41−43] In support of this notion, we found a slightly stronger inverse association between urinary TCAA concentrations and the percentage of dead spermatozoa among men with normal semen quality. Interestingly, the median urinary concentration of TCAA in our present study population (2.38 μg/L) was lower than that of men recruited from the same reproductive center in 2008 (7.40 μg/L) and 2011−2012 (7.97 μg/L). This variation within the same order of magnitude is not unexpected given the high temporal and spatial variability of DBP concentrations in the water distribution system, changes in industrial activities and water-system treatment, and considerable within-individual and between-individual variation in urinary TCAA elimination.[44,45]

The positive associations between urinary TCAA concentrations and tail% and TDM observed in our study were slightly stronger among nonsmokers and men who had normal semen quality. In addition, we also found evidence that urinary TCAA concentrations were positively associated with the percentage of dead spermatozoa among nonsmokers and negatively associated with the percentage of viable cells among nonsmokers and men with normal semen quality. Together, these findings suggest that nonsmokers and participants with normal semen quality may be more sensitive to HAA exposure, or that it may be easier to detect subtle associations among healthier men, since associations in participants with a smoking habit or abnormal semen quality may have been obscured by previous stronger risk factors (e.g., genetic predispositions, prenatal drug/chemical exposures, tobacco exposure, and occupational exposure).[46−48] Interestingly, we also found a positive association between urinary TCAA concentrations and the percentage of apoptotic spermatozoa when the "covariate-adjusted standardization plus covariate adjustment" model recommended by O'Brien was used to correct for urinary dilution,[34] suggesting the importance of controlling measurement error bias caused by variations in urinary creatinine concentrations. More studies are needed to verify these novel findings.

The underlying biological mechanisms for the positive associations between urinary TCAA concentrations and spermatozoa DNA damage could be partly explained by oxidative stress, which is the leading cause of impaired sperm DNA integrity.[49−52] Both in vivo and in vitro experiments have demonstrated that HAAs can include oxidative stress either through activating the NRf2-mediated signaling pathway,[15,53] or by inducing lipid peroxidation, glutathione peroxidase, and reactive oxygen species (ROS).[54,55] In humans, exposure to higher levels of HAAs has been also associated with higher urinary concentrations of oxidative stress markers, including 4-hydroxy-2-nonenal-mercapturic acid, 8-hydroxy-2-deoxyguanosine, and 8-iso-prostaglandin F2α, among fertile men and pregnant women.[56,57] In addition to oxidative stress, HAAs may also affect pathways involving fatty acid metabolism, phosphatidylserine externalization, angiogenesis, and tissue remodeling, eventually leading to cellular damage, sperm apoptosis, and altered gene expression DNA damage.[18,58−61]

Our study has some limitations. First, exposure misclassification in our present study cannot be excluded. To reduce exposure misclassification, we repeatedly measured urinary TCAA concentrations for the majority of study participants. The estimated intraday ICCs of urinary TCAA concentrations in our present study were equal to or greater than 0.74, which were much higher than our previous findings among 11 men who repeatedly provided 529 spot urine samples on 8 days over 3 months (ICCs = 0.09−0.23)[45] and 1760 pregnant women who provided 4165 urine samples during early, mid-, and late pregnancy (ICCs = 0.10−0.11).[24] The variation of results could be partly explained by the differences in population characteristics, study design, and sampling strategy. For instance, our present study only collected two spot urine samples at least 2 h apart within a single day, which ignored the variability in urinary TCAA concentrations that contributed to both day-to-day changes and monthly trends in HAA exposure. Second, residual confounding from unmeasured covariates cannot be fully excluded. For instance, we did not collect data on diet, air pollution, and other DBPs (e.g., trihalomethanes), which have been associated with semen quality.[62−64] Third, our study participants were recruited from a reproductive center, which may have limited the generalization of our findings to the general population due to the inclusion of more subfertile men. Fourth, given the cross-sectional design of our study, we cannot ascertain any causal relationships.

In summary, we found positive associations between urinary TCAA concentrations and the percentage of spermatozoa tail DNA and tail-distributed moment, markers of semen DNA damage. In addition, we found positive associations between urinary TCAA concentrations and the percentage of apoptotic spermatozoa when urinary TCAA concentrations were modeled as continuous variables and when we used the

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"covariate-adjusted standardization plus covariate adjustment" models to correct for urinary dilution. Our novel findings suggest that exposure to TCAA at concentrations in real-world scenarios may be associated with spermatozoa apoptosis and DNA damage.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c07725.

Comparison of population characteristics (Table S1); regression coefficients or percent changes of spermatozoa DNA damage and spermatozoa apoptosis parameters in relation to urinary TCAA concentration, stratified by smoking status and traditional sperm quality parameters (Tables S2−S5); regression coefficients or percent changes of spermatozoa apoptosis and DNA damage parameters in relation to urinary TCAA concentrations, excluding subjects whose urinary creatinine concentrations were <0.3 or >3.0 g/L or using the "covariate-adjusted standardization plus covariate adjustment" method to correct for urinary dilution (Tables S6 and S7) (PDF)

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Notes

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